MEDDOCS Open Access Publisher

Annals of Ophthalmology and Visual Sciences

Open Access | Research Article

Imaging of High- or Low-Abundance Proteins on the Rod Outer Segment Disks

Daniela Calzia[1]; Paolo Bianchini[2]; Federico Caicci[3]; Lucia Manni[3]; Silvia Ravera[4]; Carlo Enrico Traverso[5]; Alberto Diaspro[6]; Isabella Panfoli[1]*

[1]Department of Pharmacy, University of Genoa, V.le Benedetto XV,3, Genova, Italy.

[2]Nanoscopy and NIC@IIT, CHT, Istituto Italiano di Tecnologia, Genova, Italy .

[3]Department of Biology, University of Padova, Padova, Italy.

[4]Department of Experimental Medicine, Human Anatomy, University of Genoa, Genoa, Italy.

[5]Clinica Oculistica, DINOGMI, University of Genova, Genoa, Italy.

[6]Nanoscopy and NIC@IIT, CHT, Istituto Italiano di Tecnologia, Genova, Italy, DIFILAB, Dipartimento di Fisica, Universita` degli Studi di Genova, Genova, Italy.

***Corresponding Author: Isabella Panfoli**

DIFAR-University of Genoa, V.le Benedetto XV,3, 16132 GENOVA, Italy.

Email: Isabella.Panfoli@unige.it

Received: Aug 15, 2021

Accepted: Sep 08, 2021

Published Online: Sep 10, 2021

Journal: Annals of Ophthalmology and Visual Sciences

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Keywords: Confocal Laser Scanning Microscopy; Disks; F_1F_o-ATP synthase; Phototransduction; Respiratory Chain Complexes; Transmission Electron Microscopy.

Abbreviations: ATP synthase; F_1F_o-ATP synthase; CLSM; confocal laser scanning microscopy; OS; Rod Outer Segment; SD; Standard Deviation; TEM; Transmission Electron Microscopy.

Abstract

Our previous proteomic and biochemical studies suggest that the respiratory chain complexes I to V are expressed in the rod Outer Segment (OS), an organelle devoid of mitochondria, specialized for visual transduction. The expression of the cited proteins was demonstrated with several imaging techniques, even though co-localization only proved significant on retinal sections.

Here we performed Immunofluorescent Confocal (CLSM) analysis on purified disks and, Transmission Electron Microscopy (TEM) immunogold analyses on bovine retinal sections. Data on co-localization of Rhodopsin and F_1F_o-ATP synthase was not significant on the isolated disks even in the presence of good labelling of the single proteins but was significant on bovine retinal sections. Indeed, the gold spheres never laid on the same discrete sites of the disk, and labelling displayed low efficiency, likely due to the hydrophobicity of the densely stacked disks, as compared to the hydrophilic antibody milieu.

Data are consistent with the notion that tagging proteins with distinct fluorophores by direct labeling for co-localization is not completely unambiguous, especially when the stoichiometry of the two proteins is greatly different. This is the case for the disk, where Rhodopsin, utilized as a reference, represents more than 80% of its protein complement. The limitations of co-localization analysis have been largely investigated, and the present data confirm that co-localization on the disks surface is poor, especially on isolated disks, likely due to an exclusion process on small vesicles.

Cite this article: Calzia D, Bianchini P, Caicci F, Manni L, Ravera S, et al. Imaging of High- or Low-Abundance Proteins on the Rod Outer Segment Disks. Ann Ophthalmol Vis Sci. 2021; 4(1): 1013.

Introduction

The photoreceptors of the vertebrate retina are specialized neurons carrying out the first step of phototransduction [1]. The rod is characterized by two different cell compartments: The Inner Limb (inner segment, IS) comprising the ellipsoid with mitochondria, perikaryon and the synaptic contact, and the Outer Limb (outer segment, OS), where a stack of about 2,000 disks, continuously renewed [2], harbor the visual pigment, Rhodopsin (Rh). The OS is embedded in the interphotoreceptor matrix [3] receiving O2 from the choroidal vessels [4].

Microscopy techniques have been used to analyse the morphological organization of rods and to identify the membrane proteins in the OS. In the past, studies with EM and Nomads Differential Interference Contrast (NDIC) were conducted on the OS [5]. Topography of the disk membranes was also imaged by AFM [6]. We have utilized TEM and Confocal Laser Scanning Microscopy (CLSM) to image isolated disks, isolated OS, and retinal sections [7,10]. We have also stained whole retinas with mitochondrial dyes (MitoTracker (MT), Rhodamine 123, and JC-1), ex-vivo showing by CLSM that labelling only involves mitochondria and OS [11,13]. In fact mitochondrial dyes specifically label actively respiring [14,15]. Overall imaging data are consistent with the biochemical data showing the operation of the whole Electron Transfer Chain (ETC) and of Tricarboxylic Acid cycle (TCA) enzymes in the OS disks [16,17]. Disks label with a number of Antibodies (Ab) against ETC complexes and F1Fo-ATP synthase, with good cross-reactivity. Data from imaging experiments on both bovine and mouse retinal sections showed the co-localization of ETC complexes and F1Fo-ATP synthase with Rh [18]. Also, MT fluorescence co-localized with Rh autofluorescence in whole retinas [19]. The present study aimed at investigating the liability of a method often utilized to assess the simultaneous presence of two transmembrane proteins, i.e. co-localization analysis by fluorescence or immunogold microscopy. This was studied on bovine isolated OS disks or retinal sections.

Materials and methods

Materials

Ficoll, protease inhibitor cocktail, salts, leupeptin, ampicillin, and other chemicals (of analytical grade) were from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water (Milli-Q; Millipore, Billerica, MA, USA) was used. Safety precautions were taken for chemical hazards in carrying out the experiments.

Osmotically intact disk preparations

Disks were prepared by Ficoll flotation [20] from OS isolated with step sucrose gradient. OS were burst for 3 h in 5% Ficoll (Sigma-Aldrich, S. Louis, MO, USA) in distilled water, with frequent agitation. Then 2 ml distilled water were layered over the suspension which was centrifuged for 2 h at 25,000 rpm in a Beckman FW-27 rotor (100,000 x g). Osmotically intact disks (1.2 mg/ml) were collected at the interface between water and Ficoll under sterile conditions. A_{280}/A_{500} ratio of samples was 1.8 ± 0.2 (average ± SD) and rhodopsin concentration, spectrophotometrically determined, 0.8 mM. Mitochondrial contamination of the purified disk fractions was negligible. Freshly prepared disks are aggregated, as we have previously shown [13]. In order to eliminate the larger aggregates, negative charges were added to the disks by passages through a needle (25 gauge) of the purified disks prior to use, after lowering the sample protein concentration to 0.5 mg/ml. Ampicillin (100 µg/ml) and 70 µg/ml leupeptin were added to distilled water and to all solutions. Contamination of isolated intact OS by IS was minimal, as evaluated by Transmission Electron Microscopy (TEM) imaging (data not shown).

Confocal laser scanning microscopy (CLSM) imaging of disks

Immunolabeling of purified disks were conducted in solution in Eppendorf vials, at room temperature by a technique that we have previously developed [13]. Briefly, disks (60 µg protein in 50 µl) suspended in 10 mM Phosphate-Buffered Saline (PBS), pH 7.3 with 150 mM NaCl (Suspension Buffer, SB) were fixed in 3% Paraformaldehyde (20 min), resuspended and incubated with 30 mM NH_4Cl (10 min). Then disks were incubated (20 min) with the two primary Antibodies (Ab): Mouse monoclonal anti-bovine Rh (Sigma Aldrich, St.Louis, MO, USA) diluted 1:500 in PBS and rabbit polyclonal Anti- FoF_1 ATP synthase β subunit, diluted 1:400. After treatment disks were resuspended in SB, and collected by centrifugation at 6,000 rpm for 2 min in Eppendorf Centrifuge (Eppendorf, Fremont, CA); The operation was conducted twice. Then disks were incubated (20 min) with the two secondary Abs (goat anti-mouse and anti-rabbit IgG Ab conjugated with Cy3 and Cy5 fluorochrome, respectively Molecular Probes), diluted 1:800 each in PBS. After washing, the final pellet was resuspended in 10 µl of Milli-Q water and put onto glass slides, covered and sealed with MOVIOL resin, at 30°C in a dry place. In controls, disks were treated with the secondary Ab only. CLSM measurements were performed at 23°C on a Leica TCS SP5-AOBS (Leica Microsystems, Mannheim, Germany) inverted confocal laser scanning microscope. Images were acquired with Leica Confocal Software using a Leica 63X PL APO N.A. 1.40 Oil Immersion objective (Leica Microsystems CMS, Mannheim, Germany). To detect the red dye (Cy3) images were collected in the spectral region 550-650 nm accordingly to reported emission spectra. To verify the signal acquired is really due to Cy3 we performed spectral analysis exciting the red dye at 543 nm and acquiring the fluorescence spectrum from 550 to 650 nm with a band width of 5 nm. To verify the signal acquired is really due to Cy5 we performed spectral analysis exciting the green dye at 650 nm and acquiring the fluorescence spectrum from 700 to 800 nm with a band width of 5 nm. Image elaboration and analysis were realized by Image J software (U. S. National Institutes of Health, Bethesda, Maryland). Incubation of disks with the secondary Ab mixture yielded negligible immunoreactivity (data not shown).

TEM microscopy and immunogold labeling on bovine retina

Bovine eyes were obtained from a local slaughterhouse. The front half of a bovine eye was excised and the vitreous humor and lens removed. The eye cup was then filled with fixative consisting of 4% paraformaldehyde and 0.1% glutaraldehyde in PBS buffer solution. After fixation (1.5 h), the retina was removed from the eye capsule, cut into small pieces, washed overnight with 50 mM NH_4Cl, dehydrated and embedded in LR White Resin and polymerized at 58°C. Ultrathin sections were placed on Formvar-coated nickel grids and used the next day for immunogold labeling. For electron microscope immunostaining of sections, the postembedding immunogold method was applied. Ultrathin sections were first treated with block solution (10% goat serum, 0.1% Tween 20, PBS 1X). Incubation with the antiserum mix composed by Anti-Rhodopsin (Sigma Aldrich, St. Louis, MO, USA) diluted 1:200 and anti ATP-synthase- β subunit (Sigma Aldrich, St. Louis, MO, USA) diluted 1:50, was performed overnight at 4°C. Antibody binding was detected using a second antibody mixture composed by: goat anti-rabbit IgG (Sigma

Aldrich, St. Louis, MO, USA) (diluted 1:100) and goat anti-mouse IgG (British BioCell International) (diluted 1:100) coupled to gold particles (10 nm diameter for anti-rabbit, and 40nm diameter for anti-mouse). Ultrathin sections were analyzed at a FEI Tecnai G^2 transmission electron microscope operating at 100KV. The images were acquired with TIA Fei software Cam, collected and typeset in Corel Draw X3.

Results

Disks were imaged by CLSM after immunohystochemical labelling by a procedure that we had developed previously, allowing to observe purified osmotically intact disks without prior embedding of the sample. Figure 1 is a CLSM image of fluorescence of Rhodopsin (Rh, red) and F_1F_o-ATP synthase (ATP synthase, green), presented with the proteins stained by indirect immunocytochemistry.

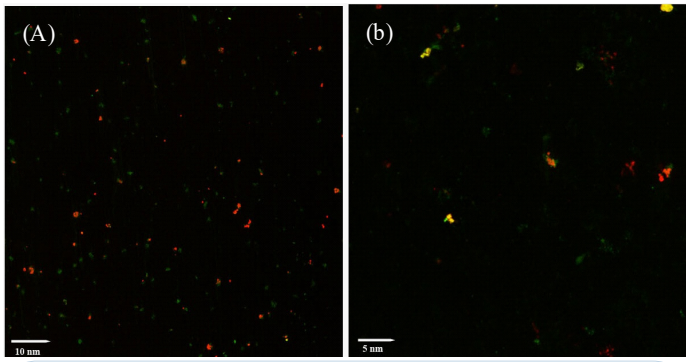

Figure 1: Co-localization of Rh and ATP synthase on purified intact disks by CLSM imaging.

Panel A. Overview of the OS disks stained with primary Ab against rhodopsin (red) and ATP synthase β subunit (green) labelled with Cy3 and Cy5- conjugated secondary antibody, respectively. Panel B. A magnification of Panel A showing that co-localization (yellow) of Rh and ATP synthase on disks ranged around 50%. The disk dimension was estimated to be about 1 µm. The images were acquired with the Leica confocal software.

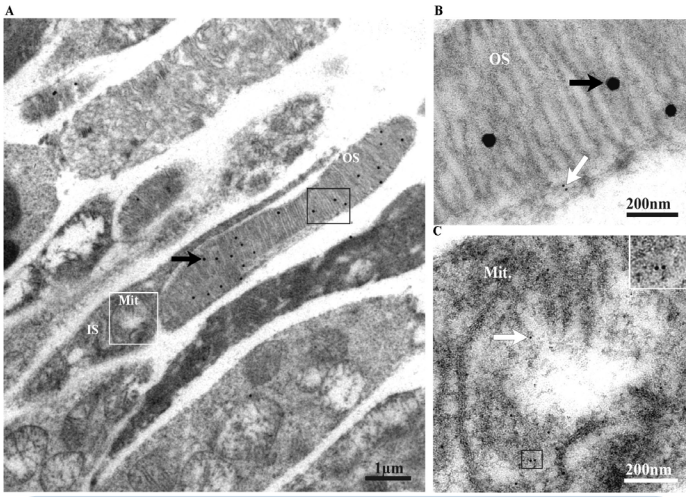

Figure 2: Transmission Electron Microscopy (TEM) on bovine retina.

A. Bovine retina double-labelled with Ab anti-Rh (large, 40 nm width gold particles) (black arrow) and anti-beta subunit of ATP synthase (small, 10 nm width gold particles). Black squared area in A is enlarged in B to show a detail of the OS, where Rh signal (black arrow) and ATP synthase signal (white arrow) co-localize. Grey squared area in A is enlarged in C, showing a magnification of an IS mitochondrion expressing ATP synthase (white arrow).

Disks appear as small aggregates of about 2-3 disks, as judged by their diameter (around 1 µm) [21]. Rh and ATP synthase are uniformly distributed on the surface of disks, similarly to what we have reported [13]. ATP synthase is a low-abundance protein, with respect to Rh, accounting for more than 80% of the disk protein complement. Panel B is a magnification of a view of Panel A, showing that co-localization analysis was not significant.

By contrast, TEM analysis of bovine eye sections shows a significant co-localization of Rh and ATP synthase on the OS. Figure 2, is a representative immunogold TEM image of a retinal section labeled with a primary Ab against the beta subunit of ATP synthase (Panel A) or Rh (panel B). Immunoreactivity is distributed on disks, not clustered in discrete locations. However, the absolute number of the gold spheres was not high as it could be expected considering the high prevalence of Rh.

Discussion

Fluorescence microscopy is routinely utilized to quantitatively assess the interaction or the co-expression of two macromolecules, by co-localization analysis [22]. This involves labeling potential interacting proteins with distinct fluorophores (for example green and red, respectively), image the tissue or organelle by direct immunofluorescence and compare the fluorescence intensity of the two fluorophores at each pixel in a two channel digital image of the sample, to reveal pixels where the two color channels overlap (yellow signal). This was done here, to image on the OS disks two proteins, i.e. Rh, a well-known maker of the the rod OS, and ATP synthase, a mitochondrial protein only recently found to be present in the disks [16,17], likely with far lower abundance. Results from CLSM experiments do not support a co-localization of Rh and ATP synthase on the isolated swollen disk vesicles, also due to the resolution of the light microscope, very close to its limit, as disk diameter ranges around 1 micrometer [23]. TEM imaging, by contrast suggests that Rh and ATP synthase proteins are both expressed in the OS. The apparent discrepancy is the result of the state of the disk sample, that was assayed in two very different conditions: in the retinal sections, where disks are native flat sacs stacked inside the OS, and in osmotically intact disks purified by a method that yields swollen disk suspensions [24]. Nonetheless, ATP synthase would be arranged inside the disk in such a functional way to be able to perform oxidative phosphorylation, together with the electron transport chain proteins to supply ATP and GTP for phototransduction [19]. Notably, absolute labelling efficiency of disk proteins is quite low due to the hydrophobic nature of the OS, filled with a stack of densely packed membranes, conceivably excluding the hydrophilic solutions in which Ab are dissolved.

Fluorescence microscopy is largely employed to determine colocalization, typically as a simple overlay of two pseudo-colored channels. For example, green and red will give rise to yellow or white spots where the two fluorophores occur in the same pixels. The overall degree of colocalization depends on labeling conditions, and on the relative proportion of the two probes that needs ideally to be nearly equal [25]. The term co-localization may mean that two fluorophores are detected in the same individual pixels due to a mere co-occurrence or due to a correlation among their relative intensities. In this respect, co-localization experiments should more appropriately be used to demonstrate an actual interaction between two molecules, rather than mere co-expression on the same subcellular site.

Conclusion

Although previous studies suggest that the OS possesses the protein complement to conduct oxidative phosphorylation [16,17], immunofluorescence CLSM analyses of isolated disks yields a good labeling with the single antibodies, where co-localization was not significant.

By contrast, TEM immunogold analyses of bovine retinal sections (Figure 2) shows that ATP synthase and Rh immunoreactivity co-localize on OS disks, even though the gold spheres are not on the same discrete sites. We have previously reported similar TEM imaging of co-localization of ETC or ATP synthase proteins with Rh in the OS [16,18]. This happens typically when automatic colocalization is done with two labeled proteins differing in total number [22], as is the case among Rh and ATP synthase. The present data demonstrate that co-localization of low abundance proteins, like those of the respiratory complexes, with high abundance ones, like Rh (accounting for 80% of the rod protein complement), can have its pitfalls (see the relative abundance of both green-and red-stained disks in Figure 1). This would also explain the fact that the presence of the proteins involved in the oxidative phosphorylation was not detected before in the many imaging studies conducted on OS and retinas: such knowledge did need a set of proteomic and biochemical data to be assessed.

References

1. Palczewski K. Chemistry and biology of the initial steps in vision: The Friedenwald lecture. Invest Ophthalmol Vis. Sci. 2014; 55: 6651-6672.
2. Finnemann SC, Bonilha VL, Marmorstein AD, Rodriguez-Boulan E. Phagocytosis of rod outer segments by retinal pigment epithelial cells requires alpha (v) beta5 integrin for binding but not for internalization. Proc Natl Acad Sci, USA. 1997; 94: 12932-12937.
3. Hollyfield JG, Rayborn ME, Nishiyama K, Shadrach KG, Miyagi M, et al. Interphotoreceptor matrix in the fovea and peripheral retina of the primate Macaca mulatta: Distribution and glycoforms of SPACR and SPACRCAN. Exp Eye Res. 2001; 72: 49-61.
4. Funk RH. Blood supply of the retina. Ophthalmic Res. 1997; 29: 320–325.
5. Hoang QV, Linsenmeier RA, Chung CK, Curcio CA. Photoreceptor inner segments in monkey and human retina: Mitochondrial density, optics, and regional variation. Vis Neurosci. 2002; 19: 395-407.
6. Buzhynskyy N, Salesse C, Scheuring S. Rhodopsin is spatially heterogeneously distributed in rod outer segment disk membranes. J Mol Recognit. 2011; 24: 483-489.
7. Panfoli I, Calzia D, Bianchini P, Ravera S, Diaspro A, et al. Evidence for aerobic metabolism in retinal rod outer segment disks. Int J Biochem Cell Biol. 2009; 41: 2555-2565.
8. Calzia D, Candiani S, Garbarino G, Caicci F, Ravera S, et al. Are rod outer segment ATP-ase and ATP-synthase activity expression of the same protein?. Cell Mol Neurobiol. 2013; 33: 637-649.
9. Calzia D, Garbarino G, Caicci F, Manni L, Candiani S, et al. Functional expression of electron transport chain complexes in mouse rod outer segments. Biochimie. 2014; 102: 78-82.
10. Calzia D, Degan P, Caicci F, Bruschi M, Manni L, et al. Modulation of the rod outer segment aerobic metabolism diminishes the production of radicals due to light absorption. Free Radic Biol Med. 2018; 117: 110-118.
11. Panfoli I, Calzia D, Ravera S, Bianchini P, Diaspro A. Immunochemical or fluorescent labeling of vesicular subcellular fractions for microscopy imaging. Microsc Res Tech. 2010; 73: 1086-1090.
12. Calzia D, Bianchini P, Ravera S, Bachi A, Candiano G, et al. Imaging of living mammalian retina ex vivo by confocal laser scanning microscopy. Anal Methods. 2010; 2.
13. Ravera S, Calzia D, Bianchini P, Diaspro A, Panfoli I. Confocal laser scanning microscopy of retinal rod outer segment intact disks: New labeling technique. J Biomed Opt. 2007; 12: 50501.
14. Baracca A, Sgarbi G, Solaini G, Lenaz G. Rhodamine 123 as a probe of mitochondrial membrane potential: Evaluation of proton flux through F (0) during ATP synthesis. Biochim Biophys Acta. 2003; 1606: 137-146.
15. Scaduto RC, Grotyohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J 1999; 76: 469-477.
16. Bruschi M, Petretto A, Caicci F, Bartolucci M, Calzia D, et al. Proteome of Bovine Mitochondria and Rod Outer Segment Disks: Commonalities and Differences. J Proteome Res. 2018; 17: 918-925.
17. Bruschi M, Bartolucci M, Petretto A, Calzia D, Caicci F, et al. Differential expression of the five redox complexes in the retinal mitochondria or rod outer segment disks is consistent with their different functionality. FASEB BioAdvances. 2020; 2: 315-324.
18. Calzia D, Oneto M, Caicci F, Bianchini P, Ravera S, et al. Effect of polyphenolic phytochemicals on ectopic oxidative phosphorylation in rod outer segments of bovine retina. Br J Pharmacol. 2015; 172: 3890-3903.
19. Calzia D, Barabino S, Bianchini P, Garbarino G, Oneto M, et al. New findings in ATP supply in rod outer segments: Insights for retinopathies. Biol Cell. 2013; 105: 345-358.
20. Smith Jr. HG, Litman BJ. Preparation of osmotically intact rod outer segment disks by Ficoll flotation. Methods Enzym. 1982; 81: 57-61.
21. Nickell S, Park PS, Baumeister W, Palczewski K. Three-dimensional architecture of murine rod outer segments determined by cryoelectron tomography. J Cell Biol. 2007; 177: 917-925.
22. Comeau JWD, Costantino S, Wiseman PW. A guide to accurate fluorescence microscopy colocalization measurements. Biophys J. 2006; 91: 4611-4622.
23. Norisuye T, Hoffman WF, Yu H. Intact photoreceptor membrane from bovine rod outer segment: size and shape in bleached state. Biochemistry. 1976; 15: 5678-5682.
24. Smith Jr. HG, Stubbs GW, Litman BJ. The isolation and purification of osmotically intact discs from retinal rod outer segments. Exp Eye Res. 1975; 20: 211-217.