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Biolistic Labeling of Retinal Ganglion Cells

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Abstract

Labeling of cellular structures is of fundamental importance in the investigation of diseases of the central nervous system. Biolistic labeling of retinal ganglion cells (RGCs) allows visualization of dendritic and synaptic structures of RGCs in retinal explants from animal models of experimental glaucoma. This technique sparsely labels RGCs, and, due to the stochastic nature of the particle delivery, all RGC types can be potentially observed in the labeled tissue. Quantification of dendritic and synaptic properties permits examination of the specific alterations to RGC morphology at different stages of degeneration, such as dendritic shrinkage and excitatory synapse loss.

Keywords

Glaucoma; Biolistic labeling; Retinal ganglion cells; Dendrites; Excitatory synapses; PSD95; Degeneration

1 Introduction

Accurate quantification of morphological properties of RGCs in retinal disease, such as soma size, dendritic complexity, and synapse density along dendritic arbors, is powerful metrics with which disease progression can be followed and functional alterations to specific retinal circuits can be predicted. Pioneer studies in primates and cats allowed identification of such cellular properties following dye injection into individual RGCs [1, 2] or into the lateral geniculate nucleus (LGN) [3]. Moreover, dye injection into individual RGCs from transgenic mice in which specific RGC populations express fluorescent protein (FP) permits the identification of RGC type-specific dendritic alterations [4]. Although fine cellular morphology can be evaluated using this method, the relative low yield of this labeling technique limits the amount of RGCs that can be analyzed in each sample and limits the analysis to cellular and dendritic morphology without providing insights on the distribution of synaptic inputs.

The DiOlistic labeling technique is an unbiased approach to labeling across RGC types, animal species, and disease models [5, 6]. Extension of this technique to deliver DNA plasmids permits exogenous expression of FP in target neurons, thus allowing biolistic labeling of dendritic structure. Co-expression of FP-tagged synaptic proteins results in targeted expression of FP in synapses within the same cell [7, 8]. In this chapter, we will describe the procedure required to label RGCs in the retina of mice and the quantification of morphological and synaptic properties of the dendritic arbor of individual RGCs [9].

2   Materials

1.   Helios gene gun system (Biorad).

2.   DNA plasmids: 1 μg/μl or higher for optimal nucleic acid concentration.

        CMV-FP for cytoplasm filling: 25 μg.

        CMV-PSD95-FP for excitatory synapse labeling: 12 μg.

3.   Spermidine: 50 μl of a 0.05 M solution.

4.   Gold particles 1.6 μm average diameter (Biorad).

5. $CaCl_2$: prepare a 1 M solution in water.

6.   ETFE tubing (0.125″ outer diameter × 0.093″ inner diameter, Saint Gobain).

7.   100% EtOH.

8.   Benchtop centrifuge and vortex mixer.

9.   Artificial mouse cerebro-spinal fluid (mACSF) for retinal explantation and culturing. Composition in mM: 119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1 mM NaH_2PO_4, 11 mM glucose, and 20 mM HEPES at room temperature; pH 7.4 adjusted with 5 M NaOH.

10.   Vectashield mounting medium (Vector).

11.   Analysis software packages: MATLAB (Mathworks), Imaris (Bitplane), ImageJ.

12.   Custom Imaris functions for synaptic puncta analysis, available at: https://github.com/lucadellasantina/imarisxt.

13.   Custom MATLAB functions for synaptic puncta analysis, available at: https://github.com/lucadellasantina/dotfinder.

3   Methods

3.1   Biolistic Labeling of Retinal Ganglion Cells

The labeling procedure consists of four fundamental steps (schematized in Fig. 1): Preparation of DNA-coated gold particles; delivery of gold particles to RGCs using the gene gun; incubation of transfected retinal explants to allow optimal expression of exogenous DNA; and finally, imaging of biolistically labeled RGC dendritic architecture and their synapses. Expression of FP in the cytoplasm will enable visualization of cell morphology under the epifluorescent microscope while co-expression of FP-tagged PSD95 will allow visualization of excitatory synapses, as the protein accumulates at RGC excitatory postsynaptic sites.

3.1.1   Preparation of DNA-Coated Gold Particles

1.   Weigh 12.5 mg gold particles and add 50 μl spermidine solution to the particles in a 1.5 ml plastic Eppendorf tube.

2. Sonicate for 15 s, add plasmids to the suspension, and spin three times in the vortex mixer.

3. Add 50 μl of 1 M CaCl$_2$ solution drop by drop while agitating at low speed (setting 5–6) on the vortex mixer.

4. Wait 15 min at room temperature to let the DNA precipitate onto the gold particles.

5. Spin the suspension for 5 s in the benchtop centrifuge.

6. Remove the supernatant and add 1.4 ml of 100% ethanol to the pellet of DNA-coated gold particles, then vortex twice to recreate a homogeneous brown suspension. Spin 6 s in the benchtop centrifuge at lowest speed.

7. Remove the supernatant and repeat ethanol washes followed by vortex and spinning three times to completely remove water residue from the ethanol suspension of gold particles.

8. The final ethanol suspension should appear uniform to the eye without any visible coarse particles or agglomerates.

3.1.2 Preparation of Tubing for Bullets

1. Cut 33 cm of ETFE plastic tubing with a razor blade.

2. Dry the inside of tubing with nitrogen gas at 3.5 psi for 10 min in the rotating device provided with the Helios gene gun system.

3. Trim the endings of the ETFE tube with a razor blade if deformed from the previous step.

4. Connect a 20 ml plastic syringe to the ETFE tube using a flexible plastic tubing adaptor.

5. Slowly load the suspension into the ETFE tube by aspirating the contents of the Eppendorf tube containing the suspension of gold particles.

6. Lay the ETFE tubing horizontally on the benchtop with the syringe still connected.

7. Let the gold precipitate onto the ETFE tube for 5 min.

8. Slowly remove the ethanol using the connected syringe. The gold particles will remain attached to the tube in a string. Optimal suction speed will leave a continuous trail of gold particles within the ETFE tube.

9. Disconnect the syringe and cover both the ends of the ETFE tube with parafilm.

10. Flick with your fingers on the tube in order to spread the gold particles inside the ETFE tube.

11. Use the cutter provided with the Helios gene gun system to create individual bullets. Collect them into a glass jar with desiccating silica gel to prevent rehydration.

12. Store bullets in the refrigerator, sheltered from light and humidity. Use within 21 days from preparation.

3.1.3 Gene Gun Preparation

1. Remove a spacer and a barrel for the gene gun from ethanol storage.

2. Dry both items of ethanol using compressed air.

3. Load the revolver into the gene gun and connect the gene gun to the gas tank.

4. Shoot several blanks to adjust the gas pressure to 40 psi.

5. Load bullets containing DNA-coated gold particles into the gene gun's barrel.

3.1.4 Particle Delivery to Tissue

1. Dissect the mouse retinas in oxygenated mACSF and place retinal whole mounts ganglion cell side up onto nitrocellulose filter paper for optimal flattening of tissue.

2. Transfer the retina attached to filter paper into an empty plastic petri dish.

3. Center the loaded gene gun over the retina.

4. Trigger the gun to project gold particles onto the tissue.

5. Transfer the retina back into oxygenated mACSF solution.

6. Incubate retinas at 37 °C degrees for ~28 h in mACSF for optimal protein expression *see* Notes 1-4.

3.2 Image Acquisition and Processing

Once RGCs have reached optimal expression of the exogenous proteins, samples can be fixed and confocal image stacks acquired for further processing (Fig. 2).

Quantification of RGC dendritic arbor's properties and excitatory synapses requires processing of confocal image stacks in three dimensions with the provided custom-written MATLAB routines.

3.2.1 Image Acquisition

1. Fix the whole mount retina in 4% paraformaldehyde for 30 min and mount the fixed tissue onto glass slides using Vectashield mounting medium.

2. Under epifluorescent illumination of the confocal microscope, locate desired RGCs using a low magnification objective (usually 10×). Then switch to a higher magnification objective for the acquisition of the confocal image stack.

3. For optimal quantification of dendritic labeling, acquire the entire dendritic arbor at a voxel size of at least $0.3 \times 0.3 \times 0.5$ μm (usually achieved using 40× or 60× objectives, depending on the RGC arbor size).

4. For optimal quantification of synaptic labeling, acquire synapses on the dendritic arbor at a voxel size of at least $0.1 \times 0.1 \times 0.3$ μm (usually achieved using a 60× objective).

5. When acquiring signal from synaptic labeling, particular attention must be paid to amplify fluorescence signal (using laser power level and photomultiplier gain) within the dynamic range of your confocal microscope acquisition system. Acquisition of oversaturated images can lead to overestimation of synaptic puncta in the following steps. We prefer acquiring image stacks at 12-bit or 16-bit sampling depth if available in your confocal acquisition system.

6. Median filter acquired image stacks using ImageJ (kernel size = 3×3 pixels) to minimize acquired thermal noise from confocal microscope photomultipliers.

7. Downsample acquired image stacks to 8-bit in case your original image stacks have higher sampling resolution. This operation will speed up further processing steps.

8. Save each color channel of the confocal image stack into an individual .TIF file for further processing.

3.2.2 Quantification of RGC Dendritic Morphology—Following the acquisition of an image stack containing the cytoplasmic FP filling and PSD95-FP synaptic labeling, skeletonization of RGC dendrites with the image analysis software Imaris will allow for quantification of dendritic arbor properties such as dendritic area and Sholl analysis of dendritic complexity.

1. Open the cell fill labeling image stack using Imaris software.

2. Using the filament function, follow the guided procedure to obtain a digital skeleton of dendrites originating from the location of the cell body (Fig. 3b).

3. Calculate accurate volume of the dendritic skeleton using the "Filament edit" tab, "process filament, diameter" function. Typical settings for mouse RGCs are: small diameter = 0.5 μm, large diameter = 20 μm, contrast = 3.

4. Retrieve Sholl analysis information using the filament properties tab, "Filament No. Sholl Intersections" and save these data for further plotting as in Fig. 3d bottom-right panel.

5. Calculate dendritic area by first calculating the polygon encompassing distal dendritic tips using Imaris function "Filament processing, Filament Convex Hull" function. Then record the area of the convex hull polygon calculated in the "Detailed statistics" tab of the polygon object.

3.2.3 Quantification of RGC Synaptic Density—The following method illustrates how to use custom Imaris and MATLAB routines to obtain a semi-automatic quantification of PSD95-FP-tagged synapses of the target RGC. The identification of synaptic puncta is first calculated based on local contrast of PSD95-FP-tagged puncta in the image stack, following the procedure described by Morgan and colleagues [7]. Then the user performs

thresholding and a final visual inspection of detected synaptic puncta candidates in the RGC-rendered volume by Imaris software.

The following puncta analysis method is sufficiently robust to be extended also to the quantification of synaptic puncta in transgenic mouse lines expressing FP-tagged synaptic proteins [10, 11] and to immunohistochemical labeling of synaptic proteins [9].

1. Install provided MATLAB functions by copying the files into your local hard drive and add the folder to your MATLAB path settings.

2. Install provided Imaris extensions by copying the files into your local Imaris extensions folder.

3. Prepare a folder structure on your computer's hard drive, encompassing a main folder called "experiment_name" containing a subfolder named "I".

4. Copy the cell fill image stack and the synaptic image stack .TIF files into "experiment_name/I" folder.

5. Open Imaris and load the dataset of the analyzed RGC obtained in Subheading 3.2.2.

6. Add PSD95 image stack to the dataset using "add channel" function present in the Imaris edit menu.

7. Export the dendritic skeleton using the custom Imaris routine "Filament, save filament as .mat" choosing "experiment_-name" folder as the target destination.

8. Export an expanded mask of RGC dendrites using the custom Imaris routine "Save filament mask," choosing "experiment_-name" folder as the target destination.

9. Open MATLAB and run the custom routines from "RunAnalysis.m" to obtain a semi-automated quantification of synaptic puncta.

10. Synaptic locations identified in the image volume, as represented in a bidimensional image projection in Fig. 3c.

11. Synaptic density will be computed and represented as bidimensional heat-maps and as linear synaptic density as a function of distance from cell soma as in Fig. 3d.

12. Plots resulting from the semi-automated analysis are available inside the "experiment_name/images" folder generated by the processing pipeline.

4   Notes

1. If labeling of RGCs is too dense, use bullets loaded with a lower density of gold particles or cut each bullet in half before repeating the experiment.

2. If labeling of RGCs is too sparse, use bullets with a higher density of gold particles.

3. If there is no labeling after incubation, confirm under a dissection microscope that the bullets were targeted correctly onto the retina. This is confirmed by the presence of shiny dots on the retinal surface observed under stereomicroscopy by direct illumination.

4. If the retina was successfully targeted by gold particles but still no fluorescent signal is present, confirm the integrity of DNA plasmids by standard transfection in cell culture (i.e., HEK-293 cells).

Acknowledgments

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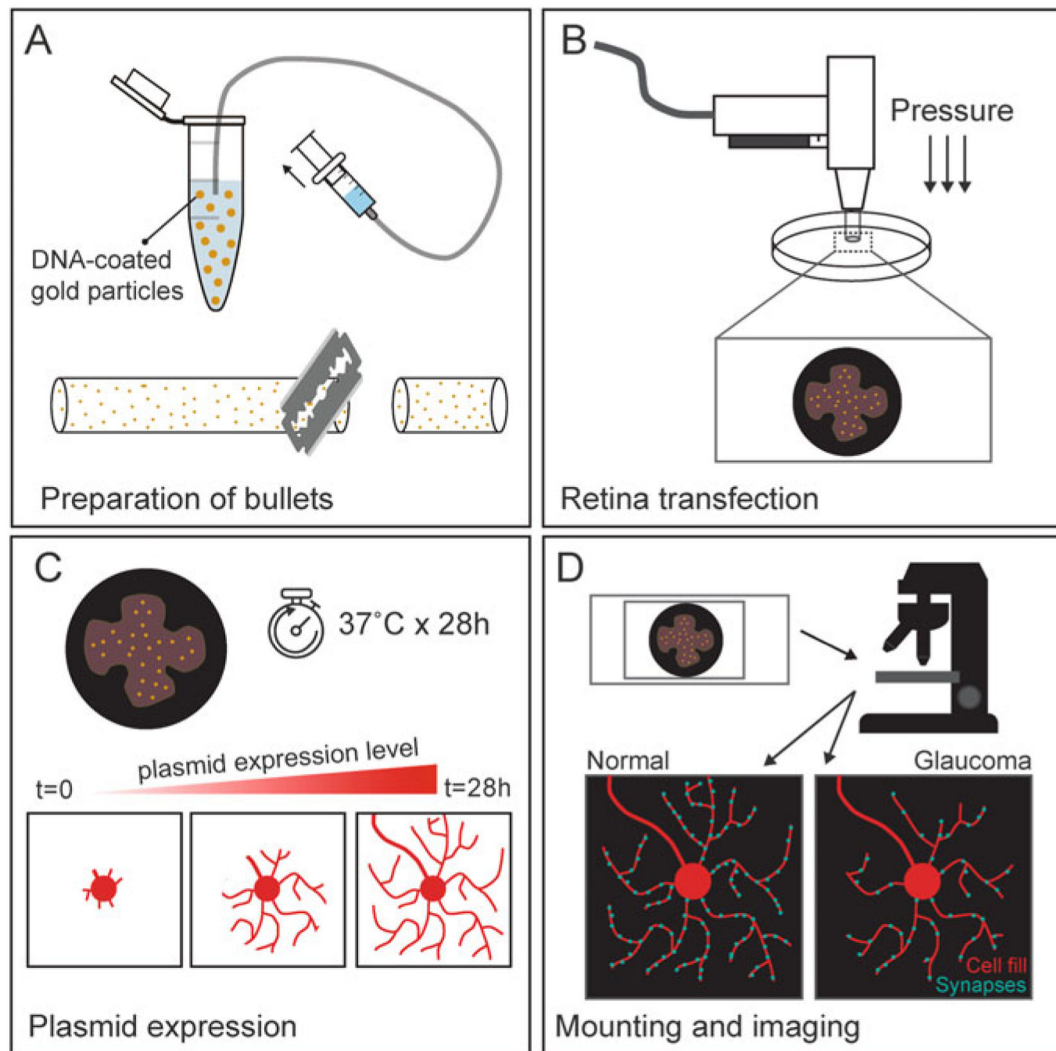
Fig. 1.
Biolistic labeling of RGCs. (**a**) Preparation of a suspension of DNA-coated gold particles in ethanol and creation of plastic bullets for transfection using the gene gun. (**b**) Biolistic transfection of RGCs using the gene gun on flat-mounted retinas. (**c**) Incubation of transfected retinas to allow optimal expression of exogenous DNA. (**d**) Mounting and confocal imaging of transfected RGCs to acquire labeling of cell morphology (*red*) and excitatory synapses (*cyan*)
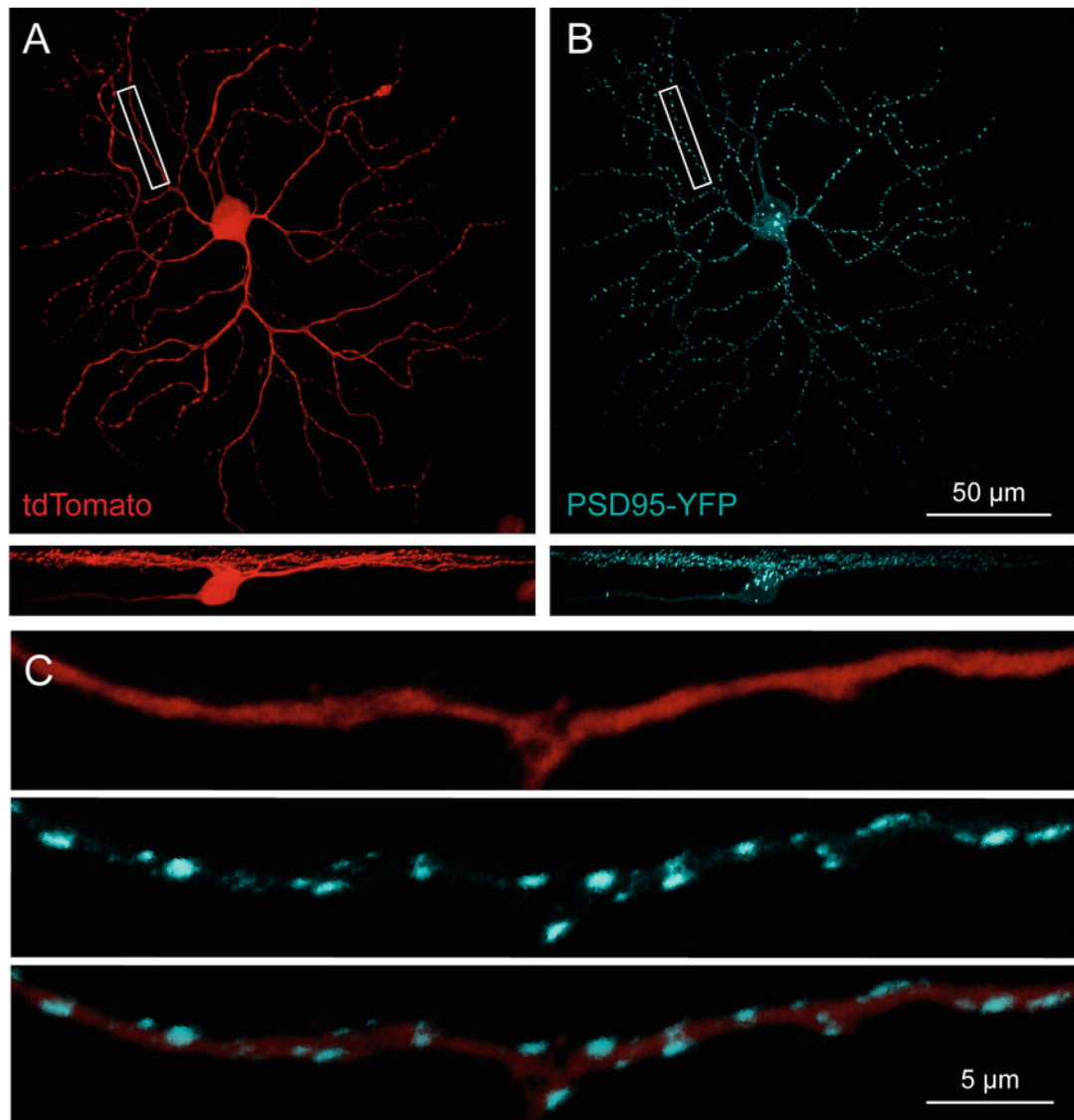
Fig. 2.
Example of a biolistically labeled RGC. (**a**) Maximum intensity projection of a biolistically labeled RGC in which cell morphology is visible by cytosolic expression of the fluorescent protein tdTomato. *Bottom panel*: side-view of the same cell. (**b**) Co-expression of the synaptic protein PDF95-YFP enables the visualization of excitatory synapses in the same RGC. *Bottom panel*: side-view of the same cell. (**c**) Magnification of the boxed area in (**a**) and (**b**) reveals complete labeling of a RGC dendritic segment (*top panel*, *red*) and excitatory synapses (*bottom panels*, *cyan*)
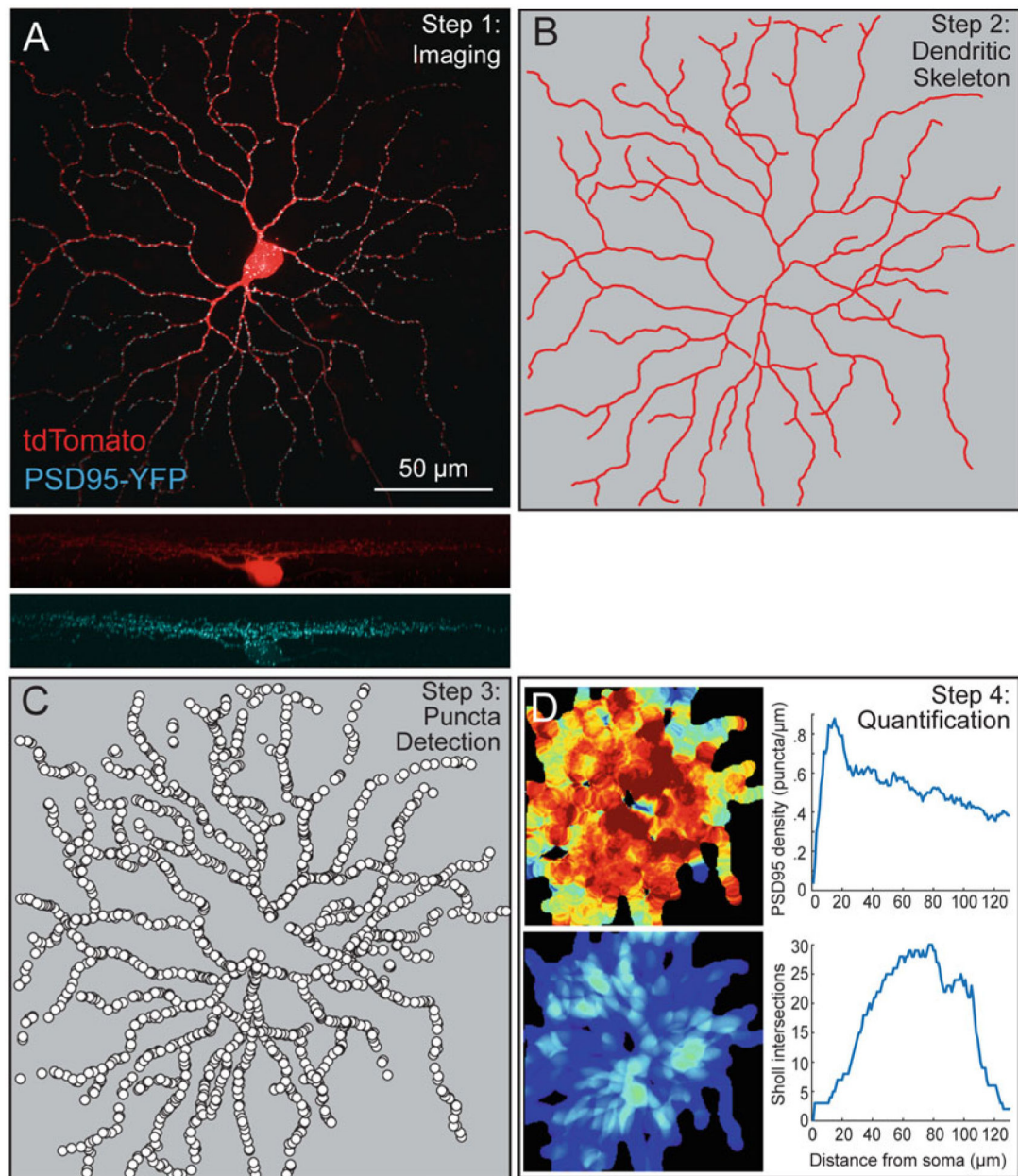
Fig. 3.
Quantification of dendritic morphology and synaptic density. (**a**) Maximum intensity projection (*en face view*) of an ON αRGC biolistically labeled to express the cytosolic fluorescent protein tdTomato and the YFP-tagged postsynaptic protein PSD95. *Bottom panels*: side view of the tdTomato and PSD95-YFP fluorescence. (**b**) Skeleton of the dendritic arbor of the RGC in (**a**) obtained by semi-automated recognition of the dendritic trajectory. (**c**) Spatial distribution of PSD95-YFP fluorescent puncta of the RGC in (**a**) obtained by semi-automated recognition of fluorescent puncta. PSD95-YFP puncta are rendered as enlarged disks in the figure for easy visual recognition. (**d**) Quantification of synaptic and dendritic properties for the RGC in (**a**). *Top-left*: Spatial representation of postsynaptic density obtained by convolving a 20 μm disk representing local PSD95-YFP

puncta density with image pixels. Hotter colors represent higher postsynaptic density. *Top-right*: Quantification of PSD95 density as a function of distance from the cell soma. *Bottom-left*: Spatial representation of dendritic density obtained by convolving a 20 μm disk representing local dendritic density with image pixels. *Bottom-right*: Sholl analysis of dendritic complexity as a function of concentric distance from the cell soma