Materials and Methods

Retina preparation

All retinal neurons in both eyes of adult New Zealand white rabbits (weight about 0.8-1.2 kg) were labeled by intraocular injection of 4μg 4,6-diamidino-2-phenylinodole (DAPI; Sigma, St. Louis, MO, U.S.A.; D-9542) under general anesthesia (intramuscular injection of 70-75 mg/kg ketamine and 15 mg/kg xylazine) 1-3 days before experiments. On experimental day, the animals were anesthesia again deeply by intramuscular injection of anesthetic with 150 mg/kg ketamine and 30 mg/kg xylazine and by intravenous injection of pure ketamine (115mg). Both eyes were then removed from the orbit and hemisected along the arc of sclera. Vitreous was removed deftly from posterior eyecup. The retina was carefully isolated from the pigment epithelium and with glass rod in modified AMES medium (only glucose and slats are preserved in the same concentration with AMES medium: NaCl, 120mM; KCl, 3.1mM; KH_2PO_4, 0.5mM; $MgSO_4$, 1.2mM; $CaCl_2$, 1.15mM; D-glucose, 6.0mM; $NaHCO_3$, 23mM). The animal was then killed by an overdose ketamine by intravenous injection.

Pieces of the DAPI-staining retina (about 5×5 mm^2) were cut and flat mounted to a cover slip coating with Cell-TAK (BD biosciences, San Jose, CA, U.S.A.; 354240), and transfer the cover clip into a chamber perfusing with modified AMES medium.

Intracellular injection

The chamber containing retina was placed on a microscope stage (Axioskop 2 FS plus, Zeiss, Oberkochen, Germany), and DAPI-labeled neurons were viewed with a Zeiss 40X plan neofluar water immersion lens and filters appropriate for DAPI and GFP (excitation, 350nm and 470nm double band pass; beam splitter, 400nm and 495nm discrete Fourier transform; emission, 460nm and 525nm double band pass, Chroma Technology Corp., Rockingham VT, U.S.A.; 51012).

Micropipettes were pulled from the thin-walled aluminosilicate filament glass tubing with filament (1.0 mm outer diameter, 0.68 mm inner diameter; Sutter Instrument Co., Novato, CA, U.S.A.; AF100-68-10) using a Flaming-Brown P97 puller (Sutter Instrument Co.) with box heat filament (Sutter Instrument Co.; FB-245B). The micropipettes were back-filled with 2% Lucifer Yellow (Sigma-Aldrich co., St. Louis, MO, U.S.A.; 861502) and 4% Neurobiotin (Vector Laboratories, Burlingame, CA, U.S.A.; SP-1120) in 0.1M Tris buffer for about 1 minute. After the tips of micropipettes were filled, the rest of micropipettes were filled with 0.1M Tris buffer and were placed into a electrode holder (WPI, Sarasota, FL, U.S.A., MEH3SW10). The holder was inserted into the amplifier probe (AM system Inc., Carlsborg, WA U.S.A.; model 1600) that was fixed on the manipulator (Narishige, MWS-31, Japan). The tip of micropipette was carefully brought into focus at the upper fringe of retina.

Single cell located at the outer two-third of the inner nuclear layer (near the sclera side) was

selected by the DAPI labeling features: brightness, size and shape. The tip of micropipette was advanced to approach the target cell through the tissue and then stabbed into the target cell by giving buzz from the amplifier. The target cell was slightly fluorescent if the tip of micropipette stabbed in. Neurobiotin and Lucifer yellow was iontophoresed into the target cell using a 10Hz biphasic current about 1-3nA for 5-30 seconds produced by function generator (Topward electronic instruments Co., Taiwan; 8120). The filling time ~~was~~ depended on the filling situation of the cell. When several cells in a piece of tissue were filled, the tissue was rinsed in 0.1M phosphate buffer (PB) for further processing.

The features for seeking the soma of blue cone bipolar cells (named BB cells in my study) in the DAPI channel: 1) The somata of the blue cone bipolar cells are located at similar position in INL with the somata of horizontal cells. 2) The somata of the blue cone bipolar have similar size with type A horizontal cells but smaller a little. 3) The shape of the somata of the blue cone bipolar is almost circular for flat view unlike the type A horizontal cells. 4) The somata of the blue cone bipolar cells are medium brightness compared with other somata at that level of the INL. Using these DAPI features really can help me for seeking the blue cone bipolar cells for microinjection. Overall, I can get one potential blue cone bipolar cell with similar morphology in dendrites in each 30~40 tries.

Peanut agglutinin (PNA) labeling of cone photoreceptors

Retinal pieces with well-injected cells were incubated in rhodamine conjugated PNA (Vector Lab., RL-1072) to stain all the cones specifically. The incubating solution was made up of 200 μg PNA and 1 mg bovine serum albumin (Sigma, A-7906) per 1 ml 0.1M PB. Incubation was 75 minutes in 1-2 ml PNA solution at the room temperature, followed by washing in 0.1M PB 3 times each 5 minutes. Postfixation for 50 minutes in 4% para-formaldehyde (Electronic Microscopy Science Co., 15710) in 0.1M PB and further washing in 0.1M PB 3 times each 5 minutes.

Immunocytochemical labeling of S cones and M cones

Blocking

The fixed retinal pieces were gently placed in the donkey blocking solution that containing 4% normal donkey serum (Jackson ImmunoResearch Laboratory, West Grove, PA, U.S.A.; 017-000-121), 0.1% Triton X-100 (Sigma, X-100) and 0.05% sodium azide (Sigma, S-2002) in 0.1M PB for 2 hours. The tissues were rinsed in 0.1M PB once.

Primary antibody labeling

The goat polyclone antiserum against the rat, mouse and human S-cone opsin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.; sc-14363) was used to identify the S-cones among the PNA- labeled cones. After blocking, the retinal pieces were incubated overnight in primary antiserum (diluted 1:200 in the donkey blocking solution) followed by further thorough washing in

0.1M PB 3 times each 10 minutes.

Secondary antibody labeling

After primary antibody labeling, the retinal pieces were incubated in FITC-conjugated donkey anti-goat IgG (Jackson Lab., 705-095-147, diluted 1:100 in blocking solution). Tissues were washed in 0.1M PB 3 times each 5 minutes.

To assess the topographical distribution of both M cones and S cones in a separate experiment, opsin labeling was also performed on the retina that had not been injected. For this I used the rabbit polyclone antiserum against the human M/L-cone opsin (Chemicon International Inc., Temecula, CA, U.S.A.; AB5405) to label the M cones (dilution 1:200). This antibody was applied in double-labeling experiments with the S-cone antibody described earlier.

Tracer visualization

To enhance the injected cell image quality, fluorescein streptavidin (Vector Lab., SA-5001, dilution 1:50 in 0.1M PB with 0.1% Triton X-100) was used overnight to visualize neurobiotin within cell after incubation in 0.1M PB with 0.1% Triton X-100 overnight. After further washing in 0.1M PB 3 times each 5 minutes, the retinal pieces were flat-mounted, vitreal side up, on glass slides and coverlipped with mounting medium (Vector Lab., H-1000). For maintaining the vertical structure of outer retina, we painted a square well on glass slide by nail polish before the mounting.

Images acquirement

Observation was made using the confocal microscopy (LSM5 Pascal laser scanning microscope; Zeiss) with a 63 X objective (Plan-Apochromat water lens, Zeiss). To identify the stratification of the injected cell in the IPL, we set the GCL uppermost boundary as 100%, followed by measuring the INL lowest boundary (0%) and the axon terminal of the cell. The stratification percentage was calculated by the depth of the axon terminal at the IPL. Images were taken in Z-stack (interval 1um) from a bipolar cell axonal terminal to its dendritic processes. Images of cone pedicles and of the S-cone distribution were taken at the same settings.

Data analysis

Fluorescence images selected for the final figures illustration and their contrast were modified by using LSM 5 image examiner (Zeiss, v3.1.0.99). We also used it to do polygon receptor field estimation and images projection whereas needed. The superimposition of S-cone distribution images with the cell dendritic processes images (Fig. 5) was made by using Adobe Photoshop CS (Adobe, San Jose, CA, U.S.A.; v8.0.1).