Feature extraction of retinal ganglion cells in the mouse retina using two-photon calcium imaging

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Abstract

Retinal ganglion cells (RGCs) comprise integral links in the computational network spanning from the initial reception of light in the retina's photoreceptors through ~30 different visual channels and into the final stages of image construction in the visual cortex. Common descriptions assume hardwired RGC networks return likewise fixed outputs given consistent stimulation, as visual information is stably and reliably represented under various light conditions and across the entire visual field. In contrast, this study will help to examine the retina's potential capacity for dynamic computation specifically with regard to RGCs, testing across stimuli including dense noise, steps of light, chirp, moving bar, and grating patterns. The dense noise and chirp stimuli were produced in *MATLAB* using *Psychtoolbox-3* for the purpose of exploring receptive field and temporal kinetics of tested cells, which are key functions for feature comparison of cell subtypes. Analysis of calcium transients indicated the presence of a variety of different cell types in the test set and will allow for further investigations of the stability of RGCs' properties while manipulating the conditions in the retina.

Introduction

Visual Processing and RGCs

Light photons are first received and processed in the photoreceptors, the sensory cells of the retina, which in turn relay chemical information to bipolar cells. Bipolar cells can then excite retinal ganglion cells (RGCs) to generate a host of diverse signals for transmission down the optic nerve to the cortex. Communication between layers is modulated by the inhibitory horizontal and amacrine cells [1].

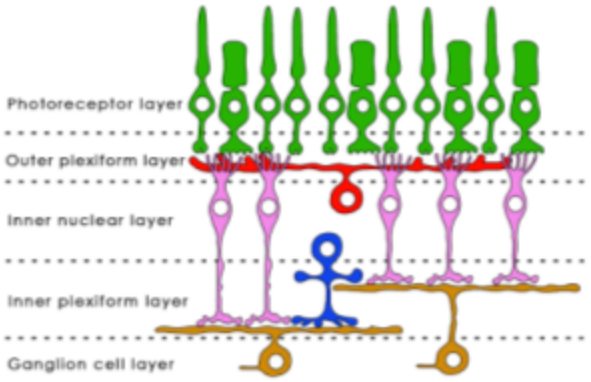

Figure 1. Schematic of retinal cell organization including photoreceptors (green), bipolar cells (pink), retinal ganglion cells (yellow), horizontal cells (red), and amacrine cells (blue).

While the role of retinal computation is not well understood in the overall context of image construction, the variety of functionality and specificity in RGCs is thought to allow for the sophisticated differentiation of optical signals prior the onset of cortical processing, although the exact extent and nature of this parallel processing is not fully known [2].

RGCs have classically been sorted based on their responses to light stimulation patterns, separated into categories differentiating between ON, OFF, and ON-OFF types as well as transient and sustained responses. Some cells have also been found to show preference with respect to direction, local edges, and uniformity of light [3-6]. Recent studies identify approximately 20 to 40 distinct types of retinal ganglion cells based on these functional factors as well as morphological differences [1,7]. Specifically, RGCs of the same type are thought to share a common physiology, morphology, connectivity, immunohistochemical profile, and genetic profile, although the exact characteristics sufficient for qualifying distinct types are still contested in many capacities [2]. For example, if axonal projections and targets were also considered type-specific, many more output channels could be defined [2]. Mouse RGCs project to at least 40 known targets and thus may come in even greater variety [8].

Calcium transients can be analyzed to gain a picture of activity in a given field of neuronal cells, revealing the morphological and functional characteristics needed to classify different RGC cell types.

The classical description of RGCs posits cells with fixed physiologies across variables including time, location, direction, and neurotransmitter levels. However, this study aims to investigate their potential capacity for dynamic computation, testing across stimuli including dense noise, steps of light, full-field chirp, moving bar, and grating patterns to distinguish differences in function both between and within cell types while changing light levels, time of the day and space across the retina.

Methods

Visual Stimuli

Visual stimulations were prepared in order to expose the retina to different patterns of light and analyze its responses. The following subsections describe 3 visual stimulations constructed in this study: (i) binary dense noise, (ii) full-field chirp, and (iii) amplitude modulated sine chirp; and 3 existing stimuli that were analyzed: (i) steps of light, (ii) moving bar, and (iii) moving gratings. Each was developed and programmed in *MATLAB* with *Psychtoolbox-3*.

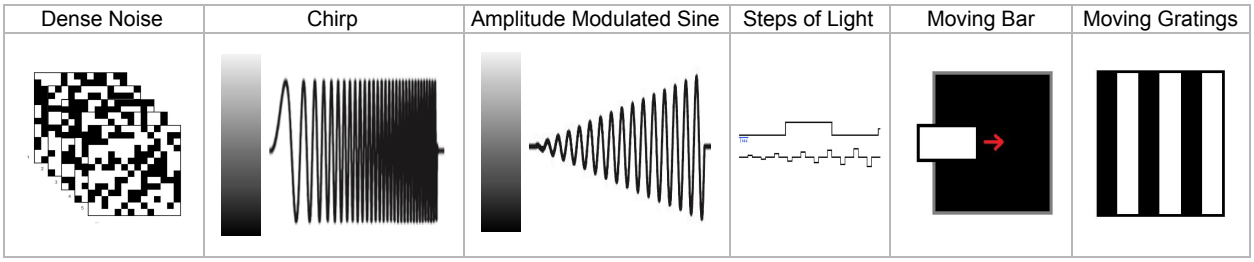

Figure 2a-f. Summary of the 6 visual stimuli employed in this study.

1. Binary Dense Noise: A visual stimulus was constructed to display binary dense noise, which can be used to estimate receptive fields [2]. A matrix of ~20μm rectangular 'pixels' change between black and white using Gaussian random numbers at a temporal frequency of 5Hz (Fig. 2a).

2. Chirp: A full-field increasing frequency 'chirp' visual stimulus can be used to characterize polarity, kinetics and the preference for temporal frequencies and contrasts [2]. Chirp, or swept-frequency cosine,

was constructed based on a function of increasing or decreasing frequency with a constant amplitude [9]. The function was transformed to achieve an amplitude of .5 centered about the line $y = .5$. The chirp stimulus displays a full screens along a grayscale, from 0 (black) to 1 (white), along the vertical axis, with time *t* along the horizontal axis (Fig. 2b).

3. Amplitude Modulated Sine: An amplitude modulated (AM) sine chirp stimulus was constructed to display a field of increasing contrast, rather than increasing frequency, which can characterize temporal kinetics of the cells. The AM chirp function takes the form of a carrier wave $v_c = V_c \sin 2\pi f_c t$ [10]. The function was transformed to have a maximum amplitude of .5 centered about the axis $y = .5$. The visual stimulus displays on the screen the color designated by a point on the y-axis, for each time *t* (Fig. 2c).

4. Steps of light: Two existing visual stimuli of steps in light levels: (i) on-off steps were used to determine the polarity preferences and latency of cells, and (ii) steps of contrasts were used to determine contrast preferences of cells (Fig. 2d).

5. Moving Bar: An existing visual stimulus that displays a white bar moving across a black screen at different angles was used to determine direction and orientation selectivity (Fig. 2e) [2]. The bar is 300μm x 900μm and moved at a speed of 500μm/s at angles of 0, 45, 90, 135, 180, 225, 270, and 315 degrees. In this study, a new analysis was introduced to determine the real time interval at which the bar was over each individual cell's field of view, which will contribute to more accurately characterizing the timing of the cells' responses to differences in the orientation of the bar.

6. Moving Gratings: A visual stimulus consisting of black and white gratings moving along the display sinusoidally (Fig. 2f) was also used to determine direction and orientation selectivity of the RGCs [2]. A similar analysis was created in this study to calculate the time intervals that each cell was exposed to the light gratings.

Tissue Preparation

The retinas were extracted from ~8-week old transgenic mice expressing GCaMP (a genetically encoded calcium indicator containing green fluorescence protein [GFP], calmodulin, and M13 sequenced from myosin light chain kinase) of either sex, who lived in normal 12 hour day/night cycles. A mouse was anaesthetized with isoflurane and killed by decapitation (by the supervising scientist), and its eyes were removed immediately. In a carboxygenated AMES solution at ~32°C, the cornea and lens were removed, the eye-cup was cut into its dorsal and ventral hemispheres, and the pigment epithelium and vitreous were removed from the retina. The retina was then flattened onto 0.22μm pore size Millipore filter paper with the GCL facing up and placed under the two-photon microscope.

Imaging

To visualize cellular activity as a real-time response to visual stimulation in a UV light ~80 cells were recorded in a 280μm x 280μm field of view under two-photon imaging. Dense noise was displayed for 5 minutes, and each visual stimulus was repeated 5 times for a total of ~30 minutes of recording. Imaging

techniques were used to record activity in specific portions of the retina, the ganglion cell layer, and for later image analysis with *ImageJ* and *MATLAB*. Activity of calcium transient levels was calculated as change in fluorescence intensity over resting fluorescence intensity while background was subtracted out (ΔF/F). Cells in the recorded field of view were manually numbered for automatic analysis of the ON/OFF, step, moving bar, and moving grating stimuli (Fig. 3a). To analyze chirp responses, cells were manually outlined in the same sequence for calculation of mean calcium transient response (Fig. 3b).

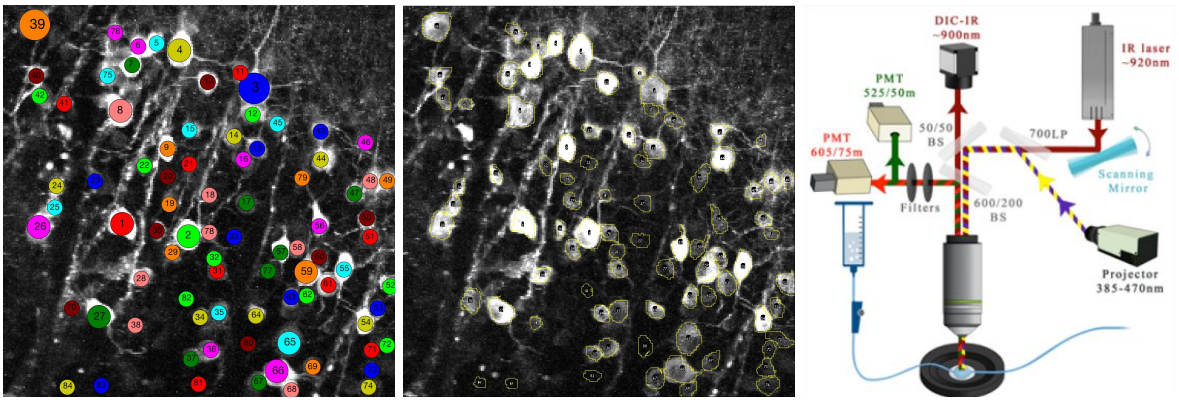

Figure 3a-c. The manually numbered (**3a**) and outlined (**3b**) cells used for data collection and analysis. Fig. **3c** summarizes the 2-photon microscope setup.

Frame Synchronization

MATLAB scripts were written to generate field of view analyses in order to coordinate imaging frames and visual stimulus frames. For the chirp, amplitude modulated sine, dense noise, and other future stimuli, this script can be used to determine what was displayed at each time point to each cell. Similarly, each frame can be saved alongside cell data to streamline the analysis process and correct the synchronisation of visual stimulations to RGC responses.

Results

Analyses were able to identify RGCs into distinct types, including ON, OFF, and ON/OFF responsive.

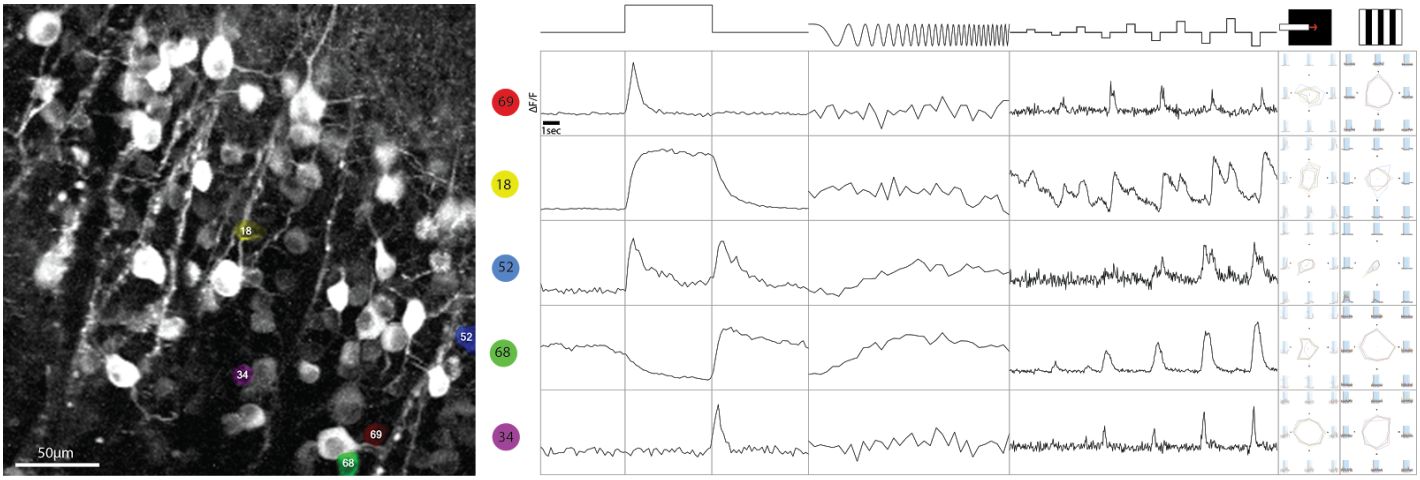

Figure 4. Responses to on/off, chirp, step, moving bar, and moving grating stimuli in cells 69, 18, 52, 68, and 34. Cell locations shown in corresponding colors at left.

ON/OFF: Responses indicate the presence of variety of different cell types, including ON transient (cell 69), ON sustained (18), ON/OFF transient (52), OFF sustained (68), and OFF transient (34) (Fig. 4).

Temporal Kinetics: Some cells, such as 69, 18, and 34 seem to exhibit approximately constant response across the duration of chirp, able to generate equivalent responses at higher frequencies although the frequencies of their responses do not appear significantly synchronous with the chirp itself. In contrast, cells 52 and 68 demonstrate what appear to be heightened or sustained responses throughout most of the experimental period, perhaps unable to distinguish between the changing colors and instead detecting a gray blend. Thus, cells displaying responses similar to 69, 18, and 34 are more likely to allow for faster recovery and continued response initiation although response frequency does not seem upwardly plastic.

Contrast Sensitivity: As the intensity of the step increased, cells 18, 52, 68, and 34 appeared to output equally increased responses, reflecting sensitivity to contrast changes within this range. However, beyond the second step, cell 69 appeared unable to modulate its response and instead returned approximately constant output regardless of changes step intensity. Thus, cell 69 reflects the fact that some cells within the test set were not found to exhibit contrast sensitivity over a significant range although most showed sensitivity over the entire range tested.

Direction and Motion Selectivity: Some cells were determined to be direction selective based on changes in their patterns of response to the moving bar and gratings with respect to direction. Most notably, cell 52 exhibited extreme directional preference as well as some variation in that preference given local (bar) vs. global (gratings) motion. Specifically, its global directional preference was even narrower, indicating the possibility for surrounding zones of inhibition.

Discussion

Cell Response Timing

One problem that previous studies have faced when determining RGCs' responses to stimuli such as the moving bar and grating patterns is the inconsistency of timing across the field of view. Since the bar, for example, moves across the entire field of view, cells in different locations of the field of view will be exposed to the bar at different times for each direction it moves in. A cell in the center of the FOV may not be affected by this, however, the timing of exposure of cells close to the edge to the stimulus such as cell 16 will vary greatly between directions. In Fig. 5a, cell 16 appears to be displaying an ON response in the top left corner, and an OFF response in the bottom right graph. In this study, a MATLAB script was written to determine the specific time intervals at which each cell was exposed to the stimulus, which can be used

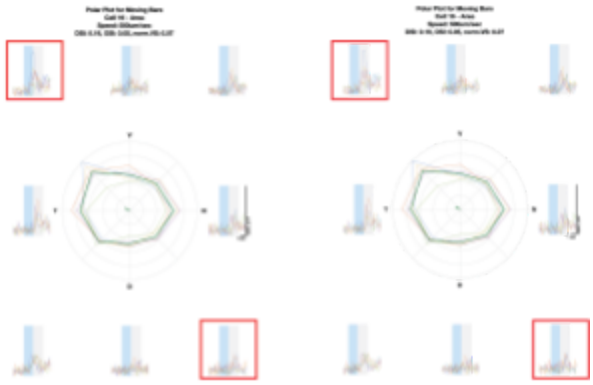

Figure 5a-b. Responses of cell 16 to 8 directions of the moving bar; **5b** is corrected for variations in timing to reveal that cell 16 displays an OFF response for each direction of the bar's motion.

to correct this problem and more accurately characterize the ON and OFF responses of each cell; in cell 16, the corrected timing in Fig. 5b reveals that it displays an OFF response to each stimulus.

Other scripts were also written to determine what was displayed at each time point for each cell, which corrected for differences in frame rate between the stimulation and imaging methods. These generic written scripts can be used for future visual stimulations to more accurately characterize each cell's responses in accordance with what caused each response.

Future Work

Dense noise analysis is still in the process of development; however, the data is expected to assist in the elucidation of receptive fields. In addition, more quantitative analysis for information extraction of cells' responses to the chirp stimulus are under revision and a more thorough analysis of results is expected to be possible in the near future.

After calibrating the new visual stimuli parameters and feature extraction of the data created in this study, more complex future experiments can explore pharmacological manipulation of the retina's condition. By implementing different clustering algorithms, dynamic changes in the response properties of different cell subtypes can be further investigated.

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