Assessment of the ipRGC Contribution to the Human Pupillary Light Reflex Using a Commercial Pupillometer

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

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2015

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Abstract

Intrinsically photosensitive retinal ganglion cells (ipRGCs) are a rare subgroup of retinal ganglion cells that contain the photopigment melanopsin. These cells are irradiance detectors that send information to the brain to control circadian rhythms and pupil size. ipRGCs mediate the sustained pupil response occurring post-illumination, due to their prolonged firing post-light offset. They are most sensitive to light with a wavelength of 480 nm. Their unique temporal properties have been used previously to assess the ipRGC contribution to human pupillary light reflex (PLR) by demonstrating a difference in the post-illumination pupil response between flickering red and blue light. My goal for this study was to determine if a commercial pupillometer, the RAPDx (Konan Medical), is capable of detecting the ipRGC contribution to the human PLR.

A total of 13 healthy subjects (ages: 23-30) were presented with three types of flickering (0.1 Hz) stimuli: red light only, blue light only, and alternating red and blue light. The stimuli were presented using the RAPDx, which recorded pupil size under infrared illumination. Pupil size was captured every 25 milliseconds by the RAPDx pupillometer. Pupil fluctuation (constriction and re-dilation) in responses to the light stimuli were recorded and analyzed.

First, the pupil responses to red and blue flashing light stimuli were assessed and compared. Inspection of the pupil traces over time revealed a gradual separation of the pupil responses to the blue and red stimuli to both monocular and binocular stimuli. The
separation was statistically significant for the comparison of the binocular, but not monocular, tests. Furthermore, there was a significant difference in the pupil responses to the blue light, but not to the red light, when the stimuli were applied binocularly versus monocularly.

After analysis of the monochrome tests, the data from the alternating pupil tests were examined. There was no significant change over time (comparing initial and final 10 seconds of test) in the difference between the pupil responses to the red versus blue stimuli in the monocular alternating test, but there was a significant change in the binocular alternating test. We then wanted to investigate whether it was the pupil responses to the blue or red pulses (or both) that were changing in response to the alternating stimuli, as compared to the monochrome stimuli. Our results suggest that the pupil responses to red light are being altered by the blue light pulses to a greater extent than the effect of the red light on the responses to blue light.

In conclusion, I believe the results presented in this work demonstrate that the RAPDx pupillometer can be used as a clinically viable instrument to assess ipRGC function. The separation over time between the blue and red stimuli, especially for binocular conditions, provides significant evidence that ipRGC contributions to the PLR can be captured using this instrument. The RAPDx should prove to be a useful tool in examining ipRGC health in a clinical environment.
Dedication

This document is dedicated to my parents and my wife.
Acknowledgments

I would like to recognize Andrew Hartwick, my advisor on this work, for his guidance and assistance throughout this entire project. In addition, Patrick Shorter and Phil Yuhas have generously contributed their time and effort to aid in the completion of this project.
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Chapter 1: Introduction

_Intrinsically Photosensitive Retinal Ganglion Cells (ipRGCs)_

Until recently, rods and cones were thought to be the only photoreceptors responsible for photodetection in the eye. In 2002, it was reported that a small group of ganglion cells could directly respond to light, providing definitive evidence for the existence of a third class of photoreceptors (Berson et al., 2002). These photoreceptors, which depolarized to light after signaling from rods and cones was blocked, were termed intrinsically photosensitive retinal ganglion cells (ipRGCs). Their intrinsic photosensitivity is derived from the photopigment melanopsin, which is expressed by ipRGCs on their dendrites, axons, and somata (Hattar et al., 2002). These cells have proven to be quite different from rods and cones in both form and function.

One aspect of ipRGC function involves regulation of circadian rhythms, which are controlled by the body’s master clock located in the suprachiasmatic nucleus (SCN). The first clues hinting towards the existence of a third photoreceptor came from studies in the field of circadian biology using mice with degenerated retinas. Although these mice lacked the majority of their rods and cones, their circadian rhythms still entrained to day/light cycles (Ebihara & Tsuji, 1980; Foster et al., 1991; Provencio et al., 1994), although with a different spectral sensitivity than that of rods and cones (Yoshimura & Ebihara, 1996). Yoshimura and Ebihara observed peak sensitivity near 480 nm for
circadian photoentrainment in the retinal degenerate mice. The unique spectral sensitivity for light-regulated functions in these mice extended beyond photoentrainment as, a few years later, pupillary reflexes in rodless/coneless mice were also shown to have peak sensitivity around 479 nm (Lucas et al., 2001).

Initially, there was skepticism surrounding these findings, as it was widely believed that there must be surviving rods and cones which were mediating circadian entrainment (Do & Yau, 2010). Another possibility was that extraocular photoreceptors were influencing circadian photoentrainment, as extraretinal photodetection is known to occur in a variety of non-mammalian species (Helfrich-Forster et al., 2001; Shand & Foster, 1999; Vigh et al., 2002; Zivkovic et al., 2000). However, this mechanism was ruled out as enucleated rodents lose all circadian photoentrainment, providing evidence that intraocular signaling is required for synchronization of the circadian clock in mammals (Nelson & Zucker, 1981; Yamazaki et al., 1999).

Around this time, a novel photopigment termed melanopsin was discovered in the skin of a species of Sub-Saharan frog (Provencio et al., 1998). Melanopsin was then localized to a small subset of RGCs (1-2%) in rodents (Gooley et al., 2001; Hattar et al., 2002; Provencio et al., 2002). Based on in vitro recordings, rat RGCs expressing this novel photopigment exhibited a spectral sensitivity to light that peaked near 480 nm (Berson et al, 2002). This wavelength, which was remarkably similar to the peak sensitivity for photoentrainment and pupil constriction in retinal degenerate mice (described above), is now widely accepted as the peak absorption of melanopsin (Dacey et al., 2005; Gamlin et al., 2007). As further support for melanopsin being the ‘circadian’
photopigment that had been postulated to exist, ipRGCs were shown to project directly to the SCN (Berson et al. 2002; Hattar et al. 2002). Finally, studies on genetically modified mice that lacked melanopsin (opn4−/−), rods and cones (RGCs in these mice had normal anatomy and projections, but lacked intrinsic photosensitivity [Lucas et al., 2003]) showed that these animals exhibited no circadian photoentrainment or pupillary light reflexes (Hattar et al., 2003). Thus, the key role for melanopsin in these non-image forming functions was then firmly established. Since, ipRGCs have been found in many different mammals (Hannibal et al., 2002; Hannibal et al., 2004; Hoshi et al., 2009; Jusuf et al, 2007; Semo et al, 2005) including humans (Dacey et al., 2005).

ipRGCs serve as irradiance detectors that are responsible for driving many light-regulated physiological processes that are distinct from pattern vision. Major targets of their axonal projections are the SCN (regulation of circadian rhythms), the olivary pretectal nucleus (control of the pupillary light reflex), and the intergeniculate leaflet (integration of circadian cues) (Gooley et al., 2003; Hannibal & Fahrenkrug, 2004; Hattar et al., 2006; Hattar et al., 2002). Many other secondary targets are innervated by ipRGCs, such as the posterior thalamic nucleus (nociception), dorsal lateral geniculate nucleus (image-forming vision), lateral posterior thalamic nucleus (higher-order processing of thalamic, cortical, and visual signals), posterior limitans thalamic nucleus (detection of rapid illumination changes) and a few other minor targets (superior colliculus, ventral lateral geniculate nucleus, ventral subparaventricular zone, and the ventrolateral preoptic nucleus) (Gooley et al., 2003; Hannibal & Fahrenkrug, 2004; Hattar et al., 2006; Hattar et
ipRGCs display unique responses when stimulated with light. They display a sluggish response, spiking after a relatively long latency period that can then persist for many seconds after the light exposure (Berson et al., 2002; Dacey et al., 2005). With these unique temporal properties, ipRGCs have poor temporal resolution in differentiating flickering and continuous light stimuli (Moose et al., 2011). This is in contrast to rods and cones that respond more quickly to light onset and terminate their responses rapidly with light offset (Nikonov et al., 2006; Raport et al., 1994). As another difference, rods and cones hyperpolarize to a light stimulus, whereas ipRGCs depolarize and fire action potentials (Berson et al., 2002; Dacey et al., 2005; Hartwick et al., 2007). With increasing light intensities, ipRGCs increase their firing rate that then reduces to a steady plateau that persists for the duration of the light stimulus (Berson et al, 2002; Dacey et al, 2005). In addition to their unique temporal characteristics (sluggish response, continuous firing, and delayed offset) and spectral sensitivity (peak at 480 nm), ipRGCs are relatively insensitive to dim light as compared to rods and cones (Do et al., 2009).

As discussed previously, melanopsin photopigment is expressed on the somata, dendrites, and axons of each ipRGC (Hattar et al, 2002). Because ipRGCs are sparse, each cell must cover a large area in order to reliably capture incoming photons, giving the ipRGCs the largest dendritic spread of all RGCs (Dacey et al, 2005). The ipRGC dendrites form an overlapping network that covers the entire retina; excluding the fovea (Dacey et al, 2005). The pathway for ipRGC axons that travel to the SCN is called the
retinohypothalamic tract (RHT). Based on low conduction velocity in the RHT and anatomical evidence, it appears as though ipRGCs are unmyelinated, which is unlike conventional RGCs (Cahill & Menaker, 1989; Guldner, 1978). They also have very large somata which can be as large as 50 microns in diameters in primates (Do & Yau 2010).

ipRGCs make up about 0.2% of the total RGC population in the macaque monkey (Dacey et al., 2005) compared to 1-2.5% in rodents (Berson et al, 2002). Even though ipRGCs comprise only a small percentage of RGCs, there are 5 known subtypes. The M1 subtype is the most widely studied group of ipRGC. Rodent M1 cells have somata which are approximately 15 um in diameter and are mostly located in the GCL, although some are displaced to the INL (Do & Yau, 2010). M1 cells differ from the other subtypes in many aspects. Their dendrites stratify in the outermost layer of the inner plexiform layer (IPL), which is also referred to as the OFF sublamina because OFF bipolar cells axons terminate here (Dacey et al., 2005). Interestingly, M1 cells receive synaptic input from ON-bipolar cells (Dumitrescu et al., 2009; Hoshi et al., 2009). They have the highest melanopsin density which in turn gives them the highest intrinsic photosensitivity of all the subclasses (Do et al., 2009). Despite their relatively high sensitivity, these cells fire at relatively low rates and are prone to depolarization block (Schmidt & Kofuji, 2009; Wong et al., 2007).

M2 cells differ in many ways from the M1 subtype. M2 cells are capable of firing action potentials at a higher rate than M1 cells, but they are less photosensitive and do not produce photocurrents as large as the ones M1 cells produce (Schmidt & Kofuji, 2009). Also in contrast to M1 cells, the M2 cells stratify in the ON sublamina of the IPL,
meaning the two subtypes are probably connected to different retinal inputs. M2 cells have both slightly larger somata and dendrites in comparison to the M1 subtype, but there are fairly equal numbers of both subtypes in the rodent retina. (Baver et al., 2008; Berson et al., 2010; Hattar et al., 2006; Schmidt & Kofuji, 2009; Viney et al., 2007).

The third subtype of ipRGC, the M3 cell, is much rarer than either M1 or M2 cells. M3 cells are bistratified and have dendrites in both the ON and OFF sublamina of the IPL (Schmidt & Kofuji, 2011), which allows them to receive input from both ON and OFF bipolar cells (Zhao et al., 2014). Finally, both M4 and M5 subtypes have dendrites that arborize in the ON sublamina of the IPL (Schmidt & Kofuji, 2011). Less is known about subtypes M3-M5 compared to M1 and M2 ipRGCs.

Although ipRGCs are photosensitive themselves, they do receive synaptic input from both bipolar and amacrine cells (Belenky et al., 2003; Dumitrescu et al., 2009; Hoshi et al., 2009; Ostergaard et al., 2007; Viney et al., 2007), carrying light-mediated signals that originated in rod and cone photoreceptors. Studies in mice have shown that when input from rods, cones, and ipRGCs are eliminated, the pupillary light reflex and circadian photoentrainment are nullified (Hattar et al., 2003; Panda et al., 2003). Also, in mice with intact rods and cones but ablated ipRGCs, these functions are lost indicating that ipRGCs are a necessary conduit for rod and cone signaling to the brain (Hatori et al., 2008). However, defects in image vision are not observed after ablation of ipRGCs (Hatori et al., 2008; Goz et al., 2008; Guler et al., 2008).

Investigations on the ipRGC influence on image-forming vision have indicated subtle
contributions, such as the long described “blindsight” in blind human patients (Sanders et al., 1974).

In the IPL, conventional RGCs and ipRGCs make synaptic connection with both bipolar and amacrine cells (Belenky et al., 2003; Dumitrescu et al., 2009; Hoshi et al., 2009; Ostergaard et al., 2007; Viney et al., 2007). The ON sublamina lies closest to the ganglion cell layer and the OFF sublamina lies closest to the inner nuclear layer. Bipolar cell axons in the ON sublamina depolarize to light increment, and bipolar cells in the OFF sublamina depolarize to light decrement (Do & Yau, 2010). Conventional ON and OFF RGC dendrites are always located in the ON and OFF sublamina of the IPL, respectively (Masland, 2001). However, ipRGCs are an exception to this rule (Dumitrescu et al., 2009; Hoshi et al., 2009). Although M1 ipRGCs arborize in the OFF sublamina, they mainly receive input from ON bipolar, as do M2 cells which arborize in the ON sublamina (Dacey et al., 2005). Additionally, M2 cells have twice as many synaptic connections compared to M1 cells (Jusuf et al., 2007). It is possible that synaptic input may be more important for M2 cells as they exhibit less intrinsic photosensitivity than M1 cells (Do and Yau 2010).

**ipRGC Function and Role in the Pupillary Light Reflex**

As discussed above, ipRGCs play a major role in many non-image visual functions. In addition to the ability to entrain to external day/night cycles (the role of ipRGCs in circadian photoentrainment was described earlier in this chapter), wild type mice that are maintained in constant darkness shift their circadian clock after being
stimulated by light during their subjective night. The degree of shift depends on the intensity of the light. However, in mice lacking melanopsin, the shift is diminished, indicating that ipRGC signaling is important to this additional process that is associated with circadian rhythm regulation (Panda et al., 2002).

A contributor to sleep regulation in primates is the melatonin released from the pineal gland. Pineal gland production and secretion of melatonin increases at night and is reduced after exposure to a bright light (Lucas et. al, 1999). Melatonin is an important hormone for sleep in diurnal animals and activity in nocturnal animals. Irradiance information is detected by ipRGCs; this photic information is then sent to the SCN, which in turn regulates melatonin production and secretion from the pineal gland. A 479nm light, which closely matches peak melanopsin spectrum, has been shown to be most effective at suppressing melatonin expression (Papamichael et al., 2012).

New research has begun investigating ipRGC contributions to photophobia with individuals who have had traumatic brain injury (TBI) and migraines. In rats, ipRGCs send axons to the posterior thalamic nucleus which is responsible for nocioception (Noseda et al., 2010). Photophobia has been shown to affect up to 80% of individuals who suffer from migraines (Choi et al., 2009; Robbins & Lipton, 2010) and 50% of patients with traumatic brain injury (Bohnen et al., 1992; Craig et al., 2008). Matynia et al. (2002) demonstrated that mice with ablated ipRGCs demonstrate less aversion to light then wild type mice. They noted wild type mice show the same aversion to red, blue or white light, and mice lacking ipRGCs had no aversion to blue or white light. Investigations into ipRGCs contributions to photophobia are ongoing.
The pupillary light reflex (PLR), and the contribution of ipRGCs to the PLR, is a major focus of this thesis. The PLR serves image vision by decreasing saturation of rods and cones, increasing depth of field, and increasing resolution (Do & Yau, 2010) and can be used to assess retinal, midbrain and autonomic function (Girkin, 2003; Kawasaki, 2005). When exposed to bright light, the PLR can reduce retinal illumination by up to 1.5 log units very rapidly (Rushton, 1965). Although this reduction in retinal illumination is a small portion of the 12 log unit range of sensitivity of the retina, it provides an immediate means for light adaptation (Rushton, 1965). The PLR is characterized by an initial peak transient constriction at light onset, followed by slight pupillary dilation, which is then sustained for the remainder of the light stimulus (McDougal & Gamlin, 2010).

In the afferent limb of the pupillary reflex, photic information is then sent, via RGC axons contained in the optic nerve and optic tract, to the pretectal olivary nucleus (OPN). Neurons in the OPN send crossed and uncrossed fibers through the posterior commissure to the Edinger–Westphal nucleus on each side. Due to this anatomy, light stimulation causes the pupil to constrict in both the light-stimulated (direct response) and contralateral eye (consensual response). In the efferent limb, the preganglionic parasympathetic fibers travel with the oculomotor nerve and synapse at the ciliary ganglion. The postganglionic parasympathetic neurons then pass from the ciliary ganglion by way of the short ciliary nerves to the iris sphincter muscle (Kardon, 2011).

Evidence for the role of ipRGCs in the PLR comes from two different types of research studies. First, anatomical studies showed significant innervation from ipRGCs to
the OPN (Hattar et al., 2002). Functional evidence was provided by Lucas et al. (2001) when they demonstrated that mice lacking both rod and cone photoreceptors retained a PLR that exhibited spectral sensitivity later shown to be consistent with ipRGCs (Berson et al., 2002). In addition, it has been shown that mice lacking the gene that encodes melanopsin, opn4−/−, cannot reach a maximal pupil constriction (~80% of max; Lucas et al., 2003). These same mice lacking melanopsin are unable to maintain (during tens of seconds of light exposure) pupil constriction, reflecting a role for ipRGCs in compensating for light adaptation by rods and cones (Zhu et al., 2007). In mice lacking rods and cones, the PLR can reach a similar maximal constriction as in wild type mice (Barnard et al., 2004; Lucas et al., 2003; Panda et al., 2003; Semo et al., 2003). Lastly, mice lacking melanopsin, rods, and cones show no light-evoked pupil constriction (Hattar et al., 2003; Panda et al., 2003), showing that there are no other photoreceptors that contribute to the PLR.

The role of ipRGCs in the PLR of human and non-human primates has also been investigated. Dacey et al. (2005) observed that in primates, ipRGCs also project to the OPN. When recorded from in vitro, they maintained prolonged depolarization post-light offset that is characteristic of rodent ipRGCs. A functional role for ipRGCs in primates were first described when Gamlin and colleagues (2007) observed that the PLR persisted in macaques after pharmacological blockade of rod- and cone-driven signals through intravitreal injections. Not surprisingly, the spectral sensitivity of the PLR matched closely to that of melanopsin (480 nm). In both humans and macaques, they compared the post-illumination pupil response (maintained pupil constriction post-light offset)
elicited by pulses of short and long wavelength light. There was more prolonged post-illumination pupil constriction after the exposure to blue light compared to red light. The temporal properties and spectral sensitivity of the post-illumination response matched closely with ipRGC characteristics. Because of this, they concluded that the sustained pupil constriction, the post-illumination pupil response (PIPR), was mediated by the continued firing of action potentials in ipRGCs (Gamlin et al., 2007). As rods and cones do not exhibit these slow temporal properties, the measurement of ipRGC function by analyzing pupil re-dilation after light offset is an effective way for measuring ipRGC function.

Mure et al. (2009) characterized pupil responses to 300 second-long stimuli. They tested completely dark adapted subjects (40 min in dark), and measured the consensual pupil response while the opposite eye was stimulated with blue light (1x10^{13} photons/cm^2/sec at 480 nm). When the stimulus was presented, the pupil rapidly constricted to a minimal size which was then followed by a redilation to a tonic or sustained pupil size. The light stimulus was maintained for 300 seconds, and they noted that the pupil stabilized to a “photoequilibrium” state, which it remained at until light offset. Approximately 60 seconds elapsed before the pupil reached this state. At light offset, the pupil slowly recovers to the dilated dark adapted state (Mure et al., 2009). They also investigated the bistability (light of different wavelengths driving melanopsin from an active to an inactive state, and vice versa) of melanopsin by exposing subjects to long and short wavelength light prior to stimulus exposure. They demonstrated that previous exposure to long wavelength light increased (by 28%), while short wavelength
light decreased (by 21%) the amplitude of sustained pupil constriction. They believe this demonstrates melanopsin’s bistability in vivo.

Bistability has been examined by others and similar findings have been uncovered by Zhu and colleagues (2007). After saturating light exposure, rather than exhibiting adaptation of the PLR, retinal degenerate and wild type mice showed increased constriction of the pupils to short wavelength light exposure. However, mice lacking melanopsin could not sustain pupillary constriction under continuous bright illumination. This “photopotentiation” effect does seem to be mediated by ipRGCs because of a few factors. First, wild type mice only show photopotentiation when the stimulus is sufficiently intense (characteristic of ipRGCs). The priming stimulus must be presented to the ipsilateral eye, suggesting that the photopotentiation must occur prior to RGC fibers crossing. Finally, the effect is lost when melanopsin is knocked out (Zhu et al. 2007). Interestingly, in vitro cellular recordings have not been able to demonstrate bistability or photopotentiation in ipRGCs (Zhu et al., 2007).

McDougal and Gamlin (2010) examined the individual contributions of rods, cones, and ipRGCs to the human pupillary light reflex. They used the unique spectral sensitivities (rods – peak at 498 nm, M-cones – 533 nm, L-cones – 564 nm, ipRGCs – 480 nm; Dowling, 1987; Dacey et al., 2005) of each photoreceptor to determine which one was driving pupillary responses. They found that not only do ipRGC responses contribute at high irradiances, but they also allow for steady state pupil constriction in photopic conditions. Cones were shown to have little effect on the steady state pupil constriction at all light intensities. In contrast, rod responses adapt over time, but they do
contribute to a steady state at irradiances that are below threshold for melanopsin activity (McDougal and Gamlin, 2010). Their findings on the spectral sensitivities of the initial and steady-state PLR are in agreement with Mure et al. (2009). However, McDougal and colleagues believe that the shift in peak sensitivity is best explained by adaptation of the outer retinal inputs, and a shift to the melanopsin driven response. Mure et al. (2009) believe this shift is due to bistability of melanopsin.

McDougal and Gamlin (2010) were also the first to describe the “winner takes all” effect on the PLR. They propose that if a stimulus is below the threshold for activating ipRGCs, then the rods cones determine the spectral sensitivity of the PLR, and in return, if it is above the threshold for ipRGC activation, the spectral sensitivity is determined by melanopsin. This means that above this threshold, the melanopsin response shunts the signals from the outer retinal photoreceptors, and the melanopsin photoresponse is solely responsible for driving the PLR. Further evidence supporting this idea comes from Sekaran and colleagues (2007). They reported that when glutamate receptor blockers were injected into mice, thereby eliminating rod and cone input to ipRGCs, there was no significant difference in the PLR when stimulated with a 480 nm light. Due to the “winner take all” effect, rods are the primary drivers for the tonic human PLR when light is under ipRGC threshold (McDougal & Gamlin, 2010). Furthermore, the initial pupil response to sinusoidal light stimulation is dominated by outer retinal rod and cone photoreceptor inputs until the threshold for ipRGCs is met (Barrionuevo et al., 2014). Findings from Wong et al (2007) support the role of rods in the sustained PLR. Using multi-electrode arrays, they noted a tonic firing rate below the melanopsin
threshold which was unlike that of conventional RGCs. Cones were found to rapidly adapt to the light stimuli, and lose ~3 log units of sensitivity shortly after light onset (Wong et al., 2007).

Use of a flickering stimulus has been used to assess ipRGC contributions to the pupil response. Gooley et al. (2012) compared pupil responses using flickering light and constant light. Pupillary constriction decreased monotonically for at least 30 min during exposure to continuous low-irradiance light. Exposure to low-irradiance flickering green light (543 nm; 0.1-4 Hz) for 30 min elicited sustained pupillary constriction responses that were more than twice as great compared to continuous light. This indicates that a flickering stimulus enhances the PLR. Yuhas et al. (2014) found that the ipRGC response was most significant with a flicker stimulus of 0.1 Hz (topic discussed in more detail later). Joyce et al (2014) showed that the melanopsin mediated PIPR amplitude is independent of the inter-stimulus interval between two light pulses (up to 1024 ms), and of the temporal frequency (0.24–4.08 Hz).

**Pupillary Function and Ocular Disease**

A potential implication of understanding the ipRGC contribution to the pupillary light reflex is to investigate whether changes in their function serves as a biomarker for RGC pathology. Pupil testing can be used as a quick and non-invasive method to investigate ocular and neurological health. Pupillary reactions have been used for decades to help assess an individual for pathology either in the eye or somewhere along the visual
pathway into the brain. It is a valuable clinical tool that can be used to assess the health of structures that cannot be easily visualized.

The most common and widely used pupil evaluation technique is the swinging flashlight test. Originally developed by Levatin in 1959, the swinging flashlight test is still the most commonly used pupil test in clinical practice today (Bremner, 2004). Relative afferent pupillary defects (RAPDs) as small as 3 dB can be detected clinically using this technique (Digre, 1998). However, normal subjects may show a RAPD up to 3 dB due to natural asymmetry of pupil inputs (Kawasaki et al., 1996). Anisocoria causes RAPDs of roughly 1 dB for every 1 mm of anisocoria due to unequal luminance of the retina (Lam & Thompson, 1999). Charalel et al (2013) found a RAPD was detected with a sensitivity of 66.7% and a specificity of 82.9%, with slightly better sensitivity and specificity in subjects who had undergone cataract surgery. They determined that RAPD screening using the swinging flashlight test with neutral density filters was moderately sensitive and strongly specific for glaucoma (Charalel et al., 2013). Others have come to this conclusion as well (Kalaboukhova et al., 2007). The swinging flashlight test is quick to perform, and does not require expensive equipment. However, an examiner must be well trained to perform and analyze the test. A RAPD can be induced by off-axis illumination, causing unequal retinal bleaching (Thompson and Jiang, 1987). One major drawback to this clinical procedure is the fact that the test is comparative between the two eyes. Bilateral or symmetric disease is very difficult to determine using the swinging flashlight test.
Pupil cycle time (PCT) is a technique which has faded from relevance. It is performed by presenting a horizontal beam of light tangential to the inferior pupil margin, recording and timing 30 oscillations of the pupil, and averaging over five trials. This pupil testing technique quickly became irrelevant due to its long duration to complete, lack of a standard light intensity used for assessment, and inability to create pupillary oscillations in individuals with marked optic nerve dysfunction (Howarth et al., 2000). Overall, the swinging flashlight test is easier to administer, takes less time, and gives better results when compared to PCT (Cox, 1982). However, one advantage of the PCT over the swinging flashlight test is that the PCT does not require a comparison eye for evaluation of optic nerve function.

Infrared video pupillography (IVP) was introduced in 1958 (Lowenstein & Loewenfeld, 1958). IVP allows for very close inspection of the pupil dynamics, as many frames can be captured per second and the infrared light has no effect on pupil response. In addition, IVP can be customized to allow for different types of stimuli and presentation frequencies to aid in determining the relative contribution of the three photoreceptors to the PLR. Further analysis of these data relating to pupil size can then be completed using computer software to carefully assess pupil dynamics.

Many different retinal and neurological diseases have been investigated for their effect on ipRGCs. One recent investigation by Kardon et al. (2011), involving patients with retinitis pigmentosa (RP), compared post-illumination pupil responses when exposed to bright blue or red light. They found individuals with RP had a more sustained PIPR with blue light compared to red light. However, initial pupil responses to red (cone)
and blue (rod) stimuli are significantly reduced in patients with RP (Kardon et al. 2011). This indicates that ipRGCs may not be affected in this outer retinal disease.

The area of most interest pertaining to ipRGCs and pupil function lies in optic nerve pathology. The axons of ipRGCs are contained in the optic nerve, and therefore they are potentially vulnerable to diseases affecting the optic nerve. There are relatively few ipRGCs in the mammalian retina (Dacey et al., 2005), which suggests that there may be reduced redundancy in the pathways regulated by these photoreceptors, indicating that early ipRGC loss could be used as a sensitive marker for optic nerve disease. However, ipRGC anatomy and function seem to be relatively spared in hereditary and ischemic optic neuropathies (Herbst et al., 2013; Kawasaki et al., 2014; Nissen et al., 2015; Perganta et al., 2013).

The ocular disease that has received the most attention with respect to pupil dynamics is glaucoma. Glaucoma is a condition which is difficult to detect in its early stages, so a screener that can identify patients close to disease onset would be beneficial. Current pupil testing for glaucoma is limited to the swinging flashlight test, which usually detects interocular differences. However, glaucoma most often is a bilateral condition so an objective screener that could detect defects without a comparison eye is warranted. Many investigations into the pupillary light reflex and deficits in this system have been concluded.

Drouyer et al. (2008) used a glaucomatous rat model and saw significant reductions (50-70%) in RGC axon terminals in all structures, including the SCN.
Although glaucomatous rats were still able to entrain circadian rhythms, they did require more time to synchronize and showed variability in their activity onset. Additionally, opsin mRNAs were reduced for all three photoreceptors (Drouyer et al., 2008). Wang et al. (2008) demonstrated that melanopsin-containing RGCs were reduced in number compared to contralateral control eyes in rats with chronic ocular hypertension. It is noteworthy that they did not see any morphological changes in the ipRGCs (Wang et al. 2008). Similar results were found by de Zavalia et al. (2011), who observed decreased numbers of melanopsin-containing cells in their glaucomatous rat model. In humans with glaucoma, patients have exhibited an overall decrease in sleep efficiency and an increase in wakeful minutes during the night when compared to controls (Lanzani et al., 2012).

Pupil studies in humans have shown significant decreases in the ipRGC-mediated post-illumination pupil response (PIPR). A sustained PIPR was demonstrated in all normal subjects after exposure to a 10 second 470-nm stimulus (Kankipati et al., 2009). Using the same stimulus in glaucomatous patients, the PIPR was significantly reduced indicating damage to the ipRGCs (Kankipati et al. 2011). In addition, as the severity of glaucoma increased, there was a correlated decrease in the PIPR. Feigl et al. (2011) also found a decreased PIPR in moderate and severe glaucoma patients, but deficiencies in the PIPR of early glaucoma patients were not found. However, they still concluded that measuring ipRGC function through the PIPR could be a good indicator of progressing glaucoma.
A correlation has been reported between reduced OCT-measured thickness and a decreased PIPR (Gracitelli et al., 2014). However, they did not find a correlation between mean deviation measured with automated perimetry and the sustained pupil response like previous studies (Kankipati et al. 2011). Subjects in the Kankipati study were found to have a larger average mean deviation, and this is thought to explain the significant correlation in their findings.

Recent work done in our lab by Yuhas (2014) attempted to characterize the flicker sensitivity of the human pupillary light reflex and isolate the ipRGC contribution to the pupil response. He investigated the change in pupil dynamics by altering flicker frequencies and intensities to best develop a flicker-based testing protocol that can be used to assess the pupillary light response in human patients. It was demonstrated that a flickering red and blue light of 0.1 Hz was best suited for ipRGC function assessment. A 0.1 Hz flicker increased maximal pupil constriction to short wavelength light over time and the overall pupil size fluctuation was small (because of sustained pupil constriction post-light offset). When comparing different light intensities, a significant difference in pupil fluctuation amplitude between red and blue light was not reached until the stimuli reached 14 log units. Separation between pupil amplitude began to show around $10^{13}$ photons/cm$^2$/s, although statistical significance was not quite reached at this intensity. These results match previous investigations of intensities needed to elicit an ipRGC response. Berson et al. (2002) determined in rats that threshold retinal irradiance for ipRGC activation was about $5 \times 10^{11}$ photon/cm$^2$/s at 500 nm which corresponds to an in vivo corneal irradiance of $2 \times 10^{13}$ photons/cm$^2$/s. Interestingly, the alternation of red and
blue light provided an enhancement to both maximal constriction of the pupil and to the PIPR. This enhancement was noted for both the red and blue stimuli. When the alternated stimuli traces were separated into their individual components (red or blue), and compared to blue or red light only tests, the effect on maximum pupil constriction and sustained constriction was enhanced. This effect has been termed photopotentiation, meaning prior light exposure, either by the red or blue, enhances the overall ipRGC response to the light.

In this prior study (Yuhas, 2014), a custom pupillometer was constructed using stimuli generated by light-emitting diodes that were focused onto the eye using lenses attached to an optical bench. The pupils were video-captured under infrared light illumination, and pupil size was later determined by manually outlining the pupil in video frames using image processing software. While this custom pupillometer and data analysis technique can be used to assess research questions, its translation to the clinic would be difficult. The goal of this thesis is to determine whether the ipRGC contribution to the human pupillary light reflex can be assessed using a commercial pupillometer, the RAPDx (Konan Medical). As discussed above, it is well established the rods, cones, and ipRGCs all play a role in the pupil’s response to light. ipRGCs have many unique properties that distinguish their response to light from that of rods and cones. Their sluggish response to light onset and offset, their spectral sensitivity, and their relative insensitivity to dim lights are all unique aspects that aid in differentiating their contributions to the PLR.
Chapter 2: Methods

This study was approved by the Biomedical Sciences Institutional Review Board (IRB) at Ohio State University and all subjects granted informed consent and HIPPA research authorization. A total of 13 healthy volunteers (ages 23-30, 9/13 female) were recruited for this study. One participant was later excluded because of having experienced a recent concussion, so data were analyzed for 12 subjects in total. Each subject had a complete eye exam within the previous year and had no history of chronic eye disease, previous eye surgery, recent head trauma, or refractive error greater than +/- 6 diopters. All subjects had a best corrected visual acuity of 20/20 OD/OS or better. Two experimental sessions were performed, with only the first session analyzed for the purposes of this thesis.

Each subject was tested using the RAPDx (Figure 1A), a commercial pupillometer developed by Konan Medical. The RAPDx uses liquid-crystal displays (LCDs) to produce full-field stimuli presented to either the right or left eyes individually, or both eyes simultaneously. A septum divides the right and left LCDs located in the RAPDx to allow for monocular stimulation. The instrument is capable of producing red (maximum irradiance of 2.6x10^{12} photons/s/cm, peak of 608 nm) or blue light (maximum irradiance of 2.7x10^{12} photons/s/cm, peak of 448 nm) stimuli (Figure 1B). Images of the eyes are recorded under infrared illumination at a 40 Hz capture rate. A display screen is
mounted on the side of the RAPDx that allows the examiner to monitor patient’s fixation during testing. In addition, pupil size is graphed in real time on the same display screen. Pupil size measurements were saved on the instrument’s internal hard drive to later be extracted and analyzed.

![Figure 1](image-url)

**Figure 1.** A) An image of the RAPDx pupillometer, courtesy of KonanMedical.com. B) Spectral sensitivity and intensity generated by the LCD screens in the RAPDx pupillometer.

During the experimental session, the tests were grouped together by stimulus color. For example, all three blue tests were run in sequence (Blue OD, Blue OS, and Blue OU); however, the order of the color groupings was randomized between subjects. Each subject participated in a total of 11 tests for the experimental session, 9 of which are analyzed for the purposes of this thesis. All tests were performed undilated, as one of the
goals of this study was to determine if a clinically applicable test can detect ipRGCs function in the pupillary light reflex. In a previous set-up used in our lab, the stimulated eye would be dilated and the recorded eye was left undilated (discussed in detail later). Monocular dilation limits the clinical translatability of a given pupil test.

The RAPDx was located in a room without windows and at the start of the session, the room lights were turned off and each subject was dark adapted for five minutes to partially adapt the rods, cones, and ipRGCs (Rushton, 1963; Wong, Dunn, & Berson, 2005). Each patient then placed their forehead against the rest and attended to the green fixation cross located inside the RAPDx. The LCDs emitted a dim white light at its darkest setting, used during the initial alignment, so subjects were not completely dark adapted for the tests. Nevertheless, the pre-test timing of 5 minutes dark adaptation and 30 seconds in the RAPDx for eye alignment was kept consistent for all subjects, and for each of the 11 pupil tests in the session. Once eye alignment was completed, the test was initiated and the RAPDx began recording pupil size approximately 40 times a second. Prior to the first light pulse, the RAPDx recorded pupil size for approximately 330 ms totaling nearly 13 pupil size measurements before flashing the first light. For the tests using either red or blue light alone, the stimulus was presented for 5 seconds, followed by 5 seconds of darkness (0.1 Hz) for 1 minute in duration. The stimuli were either presented to the right eye only (Red OD and Blue OD tests), the left eye only (Red OS and Blue OS tests) or to both eyes simultaneously (Red OU and Blue OU tests). The alternating tests used the same presentation frequency, but lasted for 2 minutes to allow for 12 total pulses (6 blue, 6 red). Red light was always presented first in the alternating
tests. As with the tests involving monochrome stimuli, the alternating tests were presented to the right (Alt OD) and left (Alt OS) eye alone, and to both eyes simultaneously (Alt OU). The RAPDx captured pupil size and graphed it on the display screen positioned towards the test administrator.

Four different sequences were used for testing (Table 1). Stimulus conditions RODBOS and BODROS were not analyzed for the purposes of this study. The RODBOS strategy consisted of alternating stimulation OD by red light and stimulation OS by blue light (0.1 Hz, 2 minute duration). BODROS conditions were similar except red light was now presented OS and blue light OD. In each stimulus sequence, the right eye was always stimulated first, followed by the left eye and finally both eyes simultaneously. The entire experimental session lasted approximately 50 minutes per subject.
<table>
<thead>
<tr>
<th>Sequence Number</th>
<th># of Subjects</th>
<th>Test Order</th>
</tr>
</thead>
</table>
| 1               | 4            | 1) Blue OD, Blue OS, Blue OU  
                 |               | 2) Red OD, Red OS, Red OU  
                 |               | 3) Alt OD, Alt OS, Alt OU  
                 |               | 4) RODBOS, BODROS                                   |
| 2               | 4            | 1) Red OD, Red OS, Red OU  
                 |               | 2) Blue OD, Blue OS, Blue OU  
                 |               | 3) Alt OD, Alt OS, Alt OU  
                 |               | 4) RODBOS, BODROS                                   |
| 3               | 2            | 1) Alt OD, Alt OS, Alt OU  
                 |               | 2) RODBOS, BODROS  
                 |               | 3) Blue OD, Blue OS, Blue OU  
                 |               | 4) Red OD, Red OS, Red OU                           |
| 4               | 2            | 1) Alt OD, Alt OS, Alt OU  
                 |               | 2) RODBOS, BODROS  
                 |               | 3) Red OD, Red OS, Red OU  
                 |               | 4) Blue OD, Blue OS, Blue OU                         |

**Table 1.** Randomized test order used during session 1. Tests RODBOS and BODROS were not analyzed for the results presented in this study.

The data were extracted from the internal hard drive of the RAPDx and imported into Microsoft Excel (2007). The data obtained from the RAPDx contained the following information: test name (Blue, Red, or Alt), stimulated eye (OD, OS, or OU), number of stimulus presentations, number of pupil size measurements during each section of the test (pre, during, and post light exposure), time elapsed during each test, and pupil size measurements in pixels for both eyes at each time point. Once imported into Excel, the data was streamlined to remove unnecessary pieces of information (see Table 2). The time/frame category was altered to make each frame capture exactly 25 ms apart. The captures done by the RAPDx were not precisely 25 ms apart, but instead were either
slightly above or below 25 ms (23.4 to 31.3 ms). Additionally, the RAPDX has “dead zones” where the instrument does not record pupil size during testing. The dead zones came directly after the pre-stimulus phase, just prior to light onset, and lasted for approximately 100 ms. These two factors presented issues when the data from each subject were averaged together. Due to the non-exact time measurements and dead zones, the averaged data for time points near the dead zone intervals would not necessarily include a data point for every subject.

Table 2. Image capture of a refined RAPDx-captured data in Microsoft Excel (2007) after filtering repetitious data.

<table>
<thead>
<tr>
<th>1)Frame</th>
<th>2)Test Name</th>
<th>3)Test Seq</th>
<th>4)Stage</th>
<th>5)RightPupilPixelRadius</th>
<th>6)LeftPupilPixelRadius</th>
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<tr>
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<tr>
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</tr>
<tr>
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<td>Post</td>
<td>76.76586</td>
<td>74.00345</td>
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</table>

The pixel radius measurements were converted to pupil diameter in mm by multiplying the pixel radius by 0.063mm. In the lab, Patrick Shorter calculated the 0.063 mm conversion factor using a reference circle of known diameter. For each test, the data were graphed with the y-axis representing pupil size (diameter in mm), and the x-axis representing time from light onset (Figure 2). All pupil size data were averaged between subjects and graphed for comparison. For all comparisons, only the right pupil size was compared because interocular pupil traces displayed almost complete overlap (Figure 2D) in direct versus consensual responses, as expected.
Figure 2. A) Graph displaying change in pupil size over time when stimulated with red or blue flickering (0.1 Hz) light. Dashed lines on the x-axis indicate light stimulation. Peaks (re-dilation) occur during light off periods and valleys represent pupil constriction during light presentation. B) Fourier analysis of the data displaying peak amplitude for one subject at the frequency (0.1 Hz) of the light flicker. C) Twice the amplitude of the Fourier transform gives an estimate of the average difference in pupil size (in mm) between the peaks and valleys for the mean pupil trace. D) Pupil trace of direct and consensual response to monocular (OD) blue light showing complete overlap in the pupil responses obtained from the two eyes.
In some instances the RAPDx was not able to capture pupil size. The two contributing factors to this lost data were subject blinks and poor infrared illumination on a small number of subjects (mainly individuals with dark irides). The RAPDx encodes missed data collection as zeros in the SUM files. For analysis and graphing purposes, the zeros were replaced with blank cells in Microsoft Excel (2007). This alteration helped to avoid cells with zeros from decreasing average pupil sizes. Having blank cells results in some time points having fewer subjects to record an average from.

Next, the data was converted from the time domain into the frequency domain by Fourier analysis (Figure 2B). This provided an objective estimate of the average amplitude of the pupil fluctuation (constriction and re-dilation) to the flickering light stimuli (Figure 2C). The Fourier analysis was performed using Microsoft Excel (2007) and for each analysis 256 data points were used. One limitation of this function is that the number of data points used must be a power of two, so ‘dummy’ data had to be used in some cases to complete the series. For this ‘dummy’ data, blank cells were filled with the same pupil size measurement obtained during the preceding measurement. The amplitude was determined at the frequency of the flickering light (0.1 Hz).

Another method of analysis compared the sum of the differences between pupil traces (Figure 3). In Figure 3 below, the purple box highlights the first cycle (5 seconds on, 5 seconds off) of light stimulation, and the green box represents the last cycle. The difference between the pupil traces was calculated for each time point and then summed together (Red minus Blue) for the period covering 0 to 10 seconds ($\Delta_{10}$) and the period
covering 50 to 60 seconds ($\Delta_{60}$). These sum values were then compared using paired T-tests to assess the change in separation between the traces over time.

**Figure 3.** The difference between pupil trace for red and blue was summed for 0-10 seconds ($\Delta_{10}$, purple box) and 50-60 seconds ($\Delta_{60}$, green box). The sums of the space between the curves were then analyzed to assess separation over time.

The RAPDx simplifies the process of testing pupil size fluctuations in comparison to the previous set-up used in our lab. The previous instrument was a custom designed LED optical system. The LEDs could be controlled by a computer program (LightControl software, DiCon LED) that allows the user to alter the frequency and intensity of the light stimulus. For each experiment, the left eye was dilated to allow for maximum illumination of the retina. The right eye was recorded under infrared illumination using a Sony HD camcorder. A black curtain shielded the subject from the light produced by the computer screen.
The recordings were analyzed using ImageJ software (National Institutes of Health, Bethesda MD). Using the circle tool in ImageJ, pupil size (pixel area) was manually measured twice per second. Pupil sizes were then transferred to Microsoft Excel (2007) where the measurements prior to light exposure were set as baseline pupil size (0%). The minimum pupil size was set as maximum constriction (100%) and all other pupil captures were assigned values between the baseline and maximal constriction values.

Some of the most challenging aspects of working with the previous custom set-up are eliminated with the RAPDx. The most significant advantage of working with the RAPDx is that the instrument measures pupil size automatically, which in turn, eliminates the most time consuming portion of analysis. It also helps to reduce measuring error that could be induced by the examiner. In addition, the RAPDx can produce a binocular stimulus. Overall, the RAPDx provides a more efficient way to measure pupil fluctuations in response to light stimuli in a clinical setting.
Chapter 3: Results

The main goal of this work is to determine if a commercial pupillometer is capable of detecting ipRGC responses (increased peak constriction, post-illumination pupil constriction) in the human PLR. Yuhas (2014) was able to detect ipRGC pupillary responses using a custom designed pupillometer described earlier. A short pilot study using the previous method of pupillometry was conducted prior to investigations with the RAPDx pupillometer. During the study, pupil responses to 6 pulses of flashing (0.1 Hz) blue light \(1 \times 10^{14} \text{ photons/cm}^2/\text{s}\) and 6 pulses of flashing red light \(7 \times 10^{14} \text{ photons/cm}^2/\text{s}\) were compared. The initial pupil constriction to the two stimuli was equalized empirically in earlier pilot studies, resulting in the difference in irradiance between the two colored stimuli.

First, the same setup as Yuhas (2014) was used to measure pupil responses in a pilot study involving 4 subjects (Figure 4) that were exposed monocularly to the red and blue light stimuli described in the preceding paragraph. After applying a Fourier transform of the data, no significant difference in flicker amplitude was noted \(P = 0.055\), paired T-test) between the tests when both pupils were left undilated, but a significant difference \(P = 0.012\), paired T-test) was found when one eye was dilated and the consensual response was recorded, similar to the results found by Yuhas (2014).
Figure 4. Pupil size (expressed as % of cornea area) recorded in subjects (n = 4), using custom pupillometer (described in Yuhas, 2014), stimulated with 0.1 Hz flashing red or blue lights. A) Mean (±SEM) pupil size over time during stimulation with flashing red or blue light with one eye dilated and the consensual response recorded. B) Fourier-derived amplitudes of the pupil flicker for dilated pupil tests (individual amplitudes plus mean). C) Mean (±SEM) pupil size over time during stimulation with red or blue light with both eyes undilated. D) Fourier-derived amplitudes of the pupil flicker for undilated pupil tests (individual amplitudes plus mean), * P<0.05, NS= Not Significant, Paired T-tests. Irradiance of red and blue stimuli 7x10^14 and 1x10^14 photons/cm²/s, at 625 and 470 nm respectively. (Figure 4: Continued)
Although the custom method was successful in identifying ipRGC responses in vivo, it is not a clinically viable instrument. Manual measurement of pupil size, obtrusive design, and monocular dilation are all obstacles that prevent the custom instrument from being utilized in a clinical setting. Next, it was assessed whether differences in the pupil responses to red and blue stimuli could be detected with the RAPDx pupillometer. Although the RAPDx allows for binocular measurements, results strictly from the right eye were analyzed, as there were negligible differences between direct and consensual
responses (Figure 2D), as expected for the human PLR. All stimuli consisted of red or blue stimuli presented at the maximum irradiance allowable on the RAPDx (see Methods and Figure 1B).

Initial analysis of pupil responses to the monochrome (red or blue) stimuli (Figures 5A, C) showed no significant difference in Fourier derived amplitudes for monocular (Red OD vs. Blue OD, P=0.150; Figure 5B) or binocular stimuli (Red OU vs. Blue OU, P=.979; Figure 5D). Although a significant difference was not found using Fourier analysis of the waveform, inspection of the pupil traces over time revealed a gradual separation of the pupil responses to the blue and red stimuli (either monocular [Figure 5A] or binocular [Figure 5C] stimuli). To assess the separation over time, the difference in pupil size measured during the two tests (Red minus Blue) for the first 10 (Δ10) and last 10 (Δ60) seconds of the minute-long monocular (Figure 5E) and binocular (Figure 5F) pupil tests were compared. The separation in pupil size over time (Δ10 versus Δ60) for the Red OD and Blue OD (red minus blue) monocular pupil tests did not quite reach statistical significance (P=0.068, Paired T-test; Figure 5G). However, this separation was statistically significant for the Red OU and Blue OU binocular tests (P<0.001; Figure 5G).
Figure 5. Comparison of pupil responses to 0.1 Hz flashing red and blue monochrome light stimuli produced with the RAPDx pupillometer.** A) Traces of mean (±SEM) pupil size comparing pupil response to monocular blue and red flashing light stimuli. B) Mean (+SEM) Fourier-derived amplitude of pupil fluctuation evoked by monocular red and blue stimuli. **NS =Not significant Paired T-tests** C) Traces of mean (±SEM) pupil size comparing pupil response to binocular blue and red flashing light stimuli. **D) Mean (+SEM) Fourier-derived amplitude of pupil fluctuation evoked by binocular red and blue stimuli. **NS =Not significant Paired T-tests. **E) The area between pupil trace for monocular and binocular red and blue stimuli was calculated between 0 and 10 seconds (Δ10, purple boxes) and between 50 and 60 seconds (Δ60, green boxes). G) Mean differences (Δ10 minus Δ60) and p-values (paired T-tests) for data obtained in pupil tests involving monocular (Pair 1) and binocular (Pair 2) red and blue light stimuli.** (Figure 5: Continued)
(Figure 5: Continued)
Next, assessment of whether the pupil responses to either the red or blue light were different when applied binocularly as compared to monocularly. A comparison of the amplitudes of pupil fluctuation evoked by the red stimuli, monocular versus binocular (Figure 6A, B) did not reach statistical significance (P=0.102). However, similar analysis for the blue stimuli revealed greater constriction to the binocular stimuli, compared to monocular stimuli (Figure 6C) and a significantly difference (P=0.001) in the Fourier-derived amplitudes of pupil fluctuation (Figure 6D). Inspection of the pupil traces for either monocular or binocular stimuli (Figures 6A, C) showed that the pupil responses to the red stimuli showed a decrease in pupil constriction over time, whereas there was a slight increase in pupil constriction over time in the blue light pupil tests. This finding is consistent with a cumulative summation of photons over time by ipRGCs during the blue light pupil test, resulting in gradually more robust spiking responses from these cells.
Figure 6. Comparison of pupil responses to Red OD vs. Red OU and Blue OD vs. Blue OU test strategies.

**Blue OU test strategies.** A) Traces of mean (±SEM) pupil size, comparing pupil responses to red light presented monocularly and binocularly. B) Mean (+SEM) Fourier-derived amplitude of pupil fluctuation evoked by monocular red vs. binocular red light. **NS** = Not significant, Paired T-test C) Traces of mean (±SEM) pupil size, comparing pupil responses to blue light presented monocularly and binocularly. D) Mean (+SEM) Fourier-derived amplitude of pupil fluctuation evoked by monocular blue vs. binocular blue light. **P<0.01, Paired T-test**
After analysis of the monochrome tests, the data from the alternating pupil tests were examined. The pupil traces recorded in response to the alternating red and blue stimuli (monocular [Alt Red or Blue OD] and binocular [Alt Red or Blue OU]) can be seen in Figure 7. Because the alternating pupil tests were 2 minutes in duration (0.1 Hz, 12 total pulses), the pupil responses to just the red or blue light pulses were separated out into their individual traces. For example, the 6 red pulses of light in the alternating pupil traces correspond to the odd numbered pulses (1,3,5,7,9,11) of light in the full test condition and the 6 blue pulses correspond to the even numbered pulses (2,4,6,8,10,12). Analysis of the mean pupil size traces demonstrates almost complete overlap with red and blue stimuli. This overlap is consistent with previous results (Yuhas, 2014) that showed that pupil responses to red light show increased constriction and reduced redilation when alternated with blue light. In other words, the blue light photopotentiates the responses to the red light, so that the responses to the two stimuli become equivalent.

The change in the area between the traces over time ($\Delta_{10}$ vs. $\Delta_{60}$) for these stimuli was then analyzed as previously done for the monochrome stimuli. Monocular alternating stimuli did not produce a significant difference between the initial ten seconds and the final ten seconds ($P=0.266$), but a significant difference was noted for the binocular alternating stimulus ($P=0.035$). However, unlike the comparison for the monochrome binocular red vs. blue stimuli in which the separation became greater over the course of the test, the separation becomes significantly smaller in the comparison of the alternating red vs. blue stimuli.
Figure 7. Comparison of pupil responses to red and blue light pulses in the alternating red/blue pupil test. A) The differences between pupil traces for Alt Red OD and Alt Blue OD, and for B) Alt Red OU and Alt Blue OU, were summed for the first 10 seconds ($\Delta_{10}$, purple boxes) and the last 10 seconds ($\Delta_{60}$, green boxes). C) Mean differences ($\Delta_{10}$ minus $\Delta_{60}$) and p-values (paired T-tests) for data obtained in pupil tests involving monocular (Pair 1) and binocular (Pair 2) red and blue light stimuli in the alternating pupil tests.

Investigation of whether it was the pupil responses to the blue or red pulses (or both) that were changing in response to the alternating stimuli, as compared to the monochrome stimuli was assessed. Comparing the responses to the monocular red light,
monochrome versus alternating (Figure 8A), the difference between $\Delta_{10}$ and $\Delta_{60}$ did not quite reach statistical significance ($P=0.075$; Figure 8C). A similar comparison for the pupil responses to the monocular blue light, monochrome versus alternating (Figure 8B), revealed that they were essentially identical ($P=0.436$; Figure 8C). For the responses to the binocular red light, monochrome versus alternating (Figure 8D), the difference between $\Delta_{10}$ and $\Delta_{60}$ was statistically significant ($P=0.010$; Figure 8C). For the binocular blue light pupil test comparisons, monochrome versus alternating, there was not a significant change between $\Delta_{10}$ and $\Delta_{60}$ ($P=0.095$). These results suggest that the pupil responses to red light are being altered by the blue light pulses to a greater extent than the effect of the red light on the responses to blue light.
Figure 8. Comparison of pupil responses to red and blue light pulses in the alternating test and red and blue light pulses in the monochrome tests. A) The differences between pupil traces for Alt Red OD and Red OD, and for B) Alt Blue OD and Blue OD were summed for the first ($\Delta_{10}$, purple boxes) and last 10 seconds ($\Delta_{60}$, green boxes). C) Mean differences ($\Delta_{10}$ minus $\Delta_{60}$) and p-values (paired T-tests) for data obtained in monocular pupil tests involving red (Pair 1) or blue (Pair 2) light. D) The differences between pupil traces for Alt Red OU and Red OU, and for E) Alt Blue OU and Blue OU were summed for the first ($\Delta_{10}$, purple boxes) and last 10 seconds ($\Delta_{60}$, green boxes). F) Mean differences ($\Delta_{10}$ minus $\Delta_{60}$) and p-values (paired T-tests) for data obtained in binocular pupil tests involving red (Pair 1) and blue (Pair 2) light. (Figure 8: Continued)
A

B

C

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<th>Paired Sample Test</th>
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<th>df</th>
<th>Sig (2-tailed)</th>
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D

E

F

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Chapter 4: Discussion

The ipRGC signature contribution (increased peak constriction, sustained post-illumination constriction) to the human pupillary light reflex could be used as a valuable clinical diagnostic tool. In theory, conditions which affect retinal function, specifically retinal ganglion cell function, could be screened for using a clinically viable tool that assesses ipRGC viability. The goal of this work was to determine if a commercially available pupillometer, the RAPDx, could be used to effectively assess ipRGC function in a population of healthy subjects.

Rods, cones, and ipRGCs all contribute to the PLR and have been shown to account for complete photodetection in mammals (Hattar et al., 2003; Panda et al., 2003). The PLR is consistent in that when first exposed to light, there is an initial transient constriction, followed by a slight dilation, which is then sustained for the length of the stimulus (McDougal and Gamlin, 2010). Cones have their greatest effect on the PLR right at light onset, but quickly lose ~ 3 log units of sensitivity and contribute little to the steady state pupil constriction at all light intensities (McDougal and Gamlin, 2010; Wong et al., 2007). Rods also adapt over time, but have been shown to contribute to the steady state pupil constriction when below ipRGC sensitivity (McDougal and Gamlin, 2010). Separation of the 3 photoreceptor responses, specifically the ipRGC response, is most easily done by examining the post-illumination pupil response. Sustained firing by
ipRGCs after light offset is unique to these cells and is not a characteristic of rods and cones. The post-illumination pupil response has been shown to be mediated by ipRGCs (Gamlin et al., 2007). ipRGCs are also responsible for maximum pupil constriction, as mice lacking the gene which codes for melanopsin are capable of producing a maximal pupillary constriction of approximately 80% of what is possible with melanopsin active (Lucas et al., 2003). Although ipRGCs are responsible for driving the peak PLR, measurement of the sustained pupil response is a more isolated measure of their function.

Flickering stimuli enhance the ipRGC response to light, as demonstrated by Gooley et al (2012) when they noted that sustained pupillary responses were twice as great when a flickering stimulus was used rather than a continuous light source. Additionally, recent work by Yuhas (2014) in our lab determined that a flickering stimulus at a rate of 0.1 Hz works well for assessing ipRGC responses. He noted an increase in maximal pupil constriction over the duration of the tests, and there was less fluctuation in the pupil size as a result of sustained pupil constriction after light offset. The same frequency (0.1 Hz) were used for all stimuli in this work.

Our initial analysis of the tests using monochrome light showed no significant difference in amplitude of pupil flicker between the red and blue stimuli. This held true for both monocular and binocular stimuli. However, in our initial pilot study and the work done by Yuhas et al. (2014) using the previous method, a significant difference in mean pupil flicker amplitude was seen. The discrepancy in results to the difference in light intensity between what the RAPDx can produce (12 log units), and what was capable with the custom set-up (14 log units). These results are consistent with previous
research that indicates a retinal illuminance of $10^{11-12}$ photons/cm$^2$ ($\sim 10^{12-13}$ at corneal plane, Berson et. al, 2002) is the threshold for melanopsin activation (Dacey et al., 2005). Although, the flicker amplitudes were not significant, analysis of the space between the pupil traces ($\Delta_{10}$ vs. $\Delta_{60}$) did allow facilitate detection of ipRGC responses.

Monocular red compared to monocular blue pupil traces demonstrated a small separation in mean pupil size recordings over the 60 second test that was not significant. This separation between the red and the blue traces was significant for the data obtained in the binocular trials, suggesting that the increase in photons is producing a larger ipRGC response. It is well established that ipRGCs are most sensitive to blue light (~480 nm) and display poor temporal resolution (Berson et al., 2002; Dacey et al., 2005). The increased constriction and sustained pupil response post light offset seen with blue light compared to red light are consistent with these characteristics mentioned above. Over time, the photons are summed (due to poor temporal resolution) and you get increasing peak constriction and increased sustained constriction with blue light.

When light stimuli of the same color (Red OD vs. Red OU, Blue OD vs. Blue OU) were compared monocularly and binocularly, distinct differences were noted between the different colored stimuli. For both red conditions (OD and OU), peak constriction decreases over time and there is no increase in sustained constriction after light offset. However, both blue conditions (OD and OU) demonstrate stable or slightly increased peak constriction throughout the 60 second duration. In addition, both blue test conditions show an increased post-illumination pupil response as you move further into the test. These differences are once again consistent with ipRGC function. The Fourier
derived mean amplitudes were not significant for the red comparison (OD vs. OU), but were for blue light. This result indicates that the binocular stimulus does induce a larger ipRGC response and ipRGC assessment *in vivo* without needing to monocularly dilate a patient, which becomes important in moving to clinical evaluation of ipRGC function.

Mean pupil size traces for the alternating stimuli (OD and OU) provided results which are consistent with conclusions made by Yuhas (2014). The traces for both alternating conditions show nearly complete overlap and indicate that the red light pupil responses begin to mimic the responses to blue light.

When the difference between the red and blue traces at the start ($\Delta_{10}$) and end ($\Delta_{60}$) or the pupil test was evaluated for the monocular alternating condition, no significant difference was found. In contrast, significance was uncovered for the binocular alternating condition. This is most likely due to the fact that in the binocular condition, the initial blue pulse (2nd pulse in sequence) stimulates ipRGCs more than the monocular condition, which gives a more sustained constriction compared to red light. In the monocular presentation, there are not enough photons to stimulate ipRGCs (therefore not causing a sustained post-illumination pupil response) to create a separation between the pupil responses to the 1st red and blue pulses. In contrast, the binocular condition does create a difference in PIPR between the 1st red and blue pulses. The initial separation seen at $\Delta_{10}$ binocularly is no longer present at $\Delta_{60}$ because the red pupil responses begin to mimic the blue (photopotentiation).

Next, the pupil responses to the red and blue light in the alternating test were compared to those elicited by the two colored stimuli in the monochrome tests. Neither
of the monocular (Red OD vs. ALT Red OD, Blue OD vs. ALT Blue OD) comparisons were significant, although the red comparison was close to reaching a significant value (P=0.075). Inspection of the binocular alternating traces indicated that they changed more over time. In the final ten seconds ($\Delta_{60}$), the pupil response to the red light in alternating test (ALT Red OU) is more constricted compared to that in the monochrome test (Red OU). The change in constriction gives a significant value for the difference between these two red light-evoked responses for the first and last cycle (P=0.010). The comparison of responses to the blue stimuli does not reveal a significant difference between the Blue OU and Alt Blue OU tests (P=0.095). These results suggest that the pupil responses to red light are being altered by the blue light pulses, to a greater extent than vice versa, in the alternating pupil test (consistent with photopotentiation). One effect not seen in our results is increased peak constriction of the blue light with the alternating stimulus. This effect was seen in the results by Yuhas et al. (2014), but was not replicated here.

Decreased light intensity may be the cause of this discrepancy as photopotentiation is only seen at intensities above ipRGC threshold.

The photopotentiation effect is not fully understood and the phenomenon has not yet been replicated in vitro. The possibility of melanopsin as a bistable pigment is one possible explanation. Short wavelength light drives phototransduction and converts the active molecule to an inactive state, which then is converted back to the active form by absorption of a long wavelength photon. In our results, the effect is actually the opposite as the short wavelength light is photopotentiating the long wavelength light. Our results do not support the traditional method of photopigment bistability.
Recent investigations into glaucoma and traumatic brain injury (TBI) patients by Patrick Shorter (2015) have revealed that these individuals have altered ipRGC responses to light. Subjects with advanced glaucoma show a reduced ipRGC response, while subjects with TBI have increased ipRGC responses to light stimulus. Decreased ipRGC responses were shown in subjects with moderate to advanced glaucoma, but investigations using the RAPDx on patients with early-stage glaucoma have not been conducted. To investigate ipRGC function in early-stage glaucoma using the RAPDx, the binocular alternating stimulus would be the best testing strategy to isolate their contribution to the PLR based off of results found in this study.

The RAPDx instrument has advantages and disadvantages in comparison to the custom designed pupillometer. The main shortcoming of the instrument is the low intensity of the LCD-generated stimuli. The irradiance output is just at ipRGC activation threshold, which is why the flicker amplitudes were not significantly different between blue and red light in the monochrome tests as found by Yuhas (2014). However, comparison of $\Delta_{10}$ vs. $\Delta_{60}$ for the unique test conditions allowed for detection of ipRGC contribution to the PLR as described above. In addition, missed pupil captures caused by poor infrared illumination created major challenges in data analysis. However, the infrared illumination system has now been upgraded in the RAPDx system, so this should not be an issue going forward.

In conclusion, the results presented here demonstrate that the RAPDx pupillometer can be used as a clinically viable instrument to assess ipRGC function. The separation over time between the blue and red stimuli, especially for binocular
conditions, provides significant evidence that ipRGC contributions to the PLR can be captured using this instrument. The RAPDx should prove to be a useful tool in examining ipRGC health in a clinical environment.
References


affected, fellow non-affected and healthy control eyes. *Front Neurol, 4*, 52. doi: 10.3389/fneur.2013.00052


circadian phase shifting. Science, 298(5601), 2213-2216. doi: 10.1126/science.1076848


Provencio, I., Rollag, M. D., & Castrucci, A. M. (2002). Photoreceptive net in the mammalian retina. This mesh of cells may explain how some blind mice can still tell day from night. Nature, 415(6871), 493. doi: 10.1038/415493a


