A STEROID DRIVEN PERIPHERAL OSCILLATOR IN FAT BODY CELLS OF *RHODNIUS PROLIXUS* (HEMIPTERA)

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GENERAL ABSTRACT

Circadian clocks are central to physiology. In animals, clocks are distributed in cells and tissues throughout the organism. The specific role of these clocks in the tissues within which they reside as well as the way they communicate and interact with each other is unclear. Using immunohistochemistry and laser confocal microscopy I have investigated whether the fat body in the insect *Rhodnius prolixus* contains an autonomous circadian clock. Fat body was found to express the canonical clock protein PER in a circadian fashion *in vivo*. However, when fat body was incubated *in vitro*, PER rapidly became undetectable. A pulse of ecdysteroid, but not brain neuropeptide extract, successfully induced PER expression but not cycling. Therefore, *Rhodnius* fat body does not seem to possess a local clock. It is inferred that PER cycling in the fat body is driven by the known rhythm of ecdysteroid concentration in the haemolymph.

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CHAPTER I: GENERAL INTRODUCTION

1.1 - Living in a cyclical environment

Organisms that are better suited to a particular environment can survive and reproduce more successfully than those that are not and in this way natural selection results in species that are better adapted to their surroundings. An integral part of our habitat, although often overlooked, is the continuously cyclical nature of the environment. This is such because the Earth spins on its axis giving rise to the cycles of day and night, while also revolving around our sun and creating the cycles of seasonal change. An additional cycle is created by the moon's revolution, producing rhythmic tides. If organisms have a way of anticipating such events, it would be extremely beneficial in their development and reproduction. Those that evolve the ability to anticipate and take advantage of the environment in this way would have a better chance of passing on their genes to future generations (Woelfle et. al., 2004). In other words, they would be more efficient biological machines. This anticipatory ability is so vital, that most living organisms, across all kingdoms (except for viruses) possess and make use of it (Dunlap, 1999). Underlying this ability are endogenous circadian rhythms. The word circadian is derived from the Latin "circa diem" which means "about a day". Circadian rhythms were first observed in plants as early as the 18th century (deMairan, 1729). In animals, endogenous circadian rhythms were first studied, analyzed and described in depth in *Drosophila* (Pittendrigh and Bruce, 1957; Pittendrigh, 1954). They defined 3 essential components of circadian rhythms: (1) They free run in aperiodic conditions; (2) They possess a period of about, but not exactly, 24 hours; and (3) they are temperature compensated, meaning that across a range of physiologically relevant temperatures, increasing or decreasing the temperature will not alter the period length of the rhythm.

Since the period length of endogenous circadian rhythms is not exactly 24 hours, they must be re-synchronized to the 24 hour day-night cycle every day. This process is called entrainment and a variety of entraining signals or “Zeitgebers”, a German word meaning “time giver”, are known. For example, cycles of temperature or humidity can entrain the organism’s circadian rhythms. However, light is by far the most reliable and important external entraining agent.

1.1.1 – The vital role of circadian rhythms

These intrinsic rhythms are proposed to have evolved independently in cyanobacteria, fungi, plants, and animals (Dunlap, 1999) and are highly adaptive and extremely important to the survival of the organism and life on Earth. Furthermore, circadian rhythms affect or control a wide diversity of processes, from photosynthesis in plants to memory formation in mammals. Organization at all levels – from individual cells and tissues to organs and whole organisms need to be coordinated in order for the organism to survive and reproduce successfully. Circadian rhythms provide temporal information to all tissues, synchronizing them with each other and ultimately, synchronizing the organism with the external environment. Such circadian control has been documented universally across kingdoms, and is found to be universal in life on

Earth. It is hypothesized that this vital timekeeping mechanism evolved to tune DNA replication during scotophase, which is the dark phase in a cycle of light and darkness. This way, DNA replication could avoid the sun's radiation on the early Earth, thereby increasing its chances to replicate successfully.

Endogenous circadian rhythms were first observed in plants. It was documented that leaf folding in the *Mimosa* plant occurred at dusk, and opening of leaves prior to dawn, anticipating the sunlight and getting the plant ready for photosynthesis (deMairan, 1729). This process was found to be endogenous as the rhythm of leaf folding persisted when the plant was placed in a continuously dark cave, in aperiodic conditions. Further, in 1832, deCandolle showed that this leaf folding rhythm does not repeat with exactly 24h in continuous darkness. Further experimentation with leaves of the common bean *Phaseolus* showed that light could act as a Zeitgeber, entraining the leaf movement to exactly a 24h periodicity (Bünning, 1936). These circadian rhythms have more recently been shown to have a role beyond just leaf movement/folding. Indeed, they are found to increase reproductive fitness of plants by synchronizing flower formation with other members of the species (Green et. al., 2002; Johnson et. al., 1998). Moreover, they play a role in the physiological, developmental, and metabolic activities, in each specific phase relationship to the environmental cycle, both daily and seasonally (Webb, 2003). These circadian rhythms allow the plant to anticipate sunrise each day and thereby prepare the photosynthetic and stomatal apparatus prior to first light touching the plant, thus maximizing energy production.

It has also been shown that prokaryotes have circadian control of gene expression (Kondo and Ishiura, 2000). The circadian mechanism precisely allows expression of genes involved in photosynthesis during the day-time, and genes responsible for nitrogen fixation during the night-time. This is vital, as the two sets of genes would hinder each other's function if expressed simultaneously. As such, circadian rhythms provide this unicellular organism another way of compartmentalizing cellular processes – a temporal separation instead of a spatial separation (Kondo and Ishiura, 2000). This ability was found to aid in the fitness of the organism relative to ones with genetically disrupted circadian rhythms.

Circadian rhythms have also been documented to control the growth rate, hyphal formation, sporulation, sexual development, CO_2 metabolism, and rhythmic gene expression in fungi (Bell-Pedersen et. al., 2006; Dunlap and Loros; 2006).

In the animal kingdom, circadian rhythms also play important roles and functions. For instance, disruption of these rhythms leads to decreased lifespan in *Drosophila melanogaster* (Klarsfeld and Rouyer, 1998). Moreover, execution of ecdysis – shedding of the cuticle – and eclosion – emergence of the adult from the pupal case – rhythms are also under such control (Park et. al., 2003). Further, they have a role in mating. For example, females in some Lepidoptera species were shown to release a sex pheromone during scotophase and this rhythm was found to be under circadian regulation (Castrejón-Gómez, 2010). Indeed, circadian rhythms also have important roles in mammals as well, including humans, in higher centres of the brain, including in memory formation and persistence (Eckel-Mahan, 2012).

Thus, circadian rhythms exist in all taxa and influence nearly all biological processes. Even more importantly, they have the ability to synchronize every cell, tissue, and organ to work together to achieve a desired outcome. They accomplish this via clock cells that are able to integrate outside input (zeitgebers) such as light for entrainment, and provide rhythmic information to the rest of the organism. Virtually all organisms, including animals, plants, fungi, bacteria, now have an endogenous way to tell time, a biological clock. The cellular and molecular machinery of clock cells consists in large part of transcription translation feedback loops, and is described in more detail in section 1.2. Following this is a discussion on how clock cells are able to entrain the rest of an animal's cells, tissue and organs via rhythmic output, and to synchronize the animal to the outside environment: in mammals (section 1.2.1) and insects (section 1.2.2).

1.1.2 – Why is it important to study circadian control?

A myriad of studies now show that synchronizing our biological clock with the external environment is extremely important (Hastings et. al., 2007; Salgado-Delgado et. al., 2011). De-synchronization can lead to a plethora of illnesses and conditions, some lethal. Unfortunately, since the advent of electricity, our society now functions around the clock, requiring a number of people to work during the night, or rotating shift work. Further, travelling long distances is now more prevalent than in the past, resulting in a lot of people experiencing "jet-lag". Moreover, living in the city, people are exposed to artificial lighting during the night. All of this leads to the de-synchronization of our internal clock from the external, natural environment.

As previously mentioned, this de-synchronization can profoundly affect our well-being. It is now well documented that poor sleep patterns exhibited by shift workers can lead to impaired metabolic function and mental health (Cauter et. al., 2008), and greater risk of hypertension and gastrointestinal disturbances (Knutsson, 2003; Oishi et. al., 2005; Sookoian et. al., 2007). Worse than this is that rotating shift work leads to increased risk of developing a number of cancers (Schernhammer et. al., 2003 and 2006). Further, it enhances tumour progression (Filipski et. al., 2002 and 2004). Sleep disorders, whether caused by shift work, jet-lag, or some other alteration in the circadian machinery, are also documented to lead to mood disorders and various types of depression, including bipolar disorder (Germain and Kupfer, 2008; Dallaspezia and Benedetti, 2009). Travelling across time zones quickly leads to jet-lag, which can be manifested by various symptoms such as: fatigue, insomnia, impaired cognitive performance, gastrointestinal disturbances and irritability (Barclay et. al., 2011).

Elucidating and understanding the circadian clock mechanism can lead to useful treatment and therapies. For instance, scheduled medication for depression is found to significantly improve results over taking drugs at any time (Salgado-Delgado et. al., 2011). Timed delivery of cytotoxic therapies to cancer patients leads to improved ablation of tumour cells, with less impact on healthy ones (Levi and Schibler, 2007). This has been made possible because research in circadian rhythms showed that tumour cells still possess functional clockwork, but they are in a different phase than the wild type host cells (Filipski et. al., 2004; Davidson et. al., 2006). The above treatments are known as chronotherapies and are of great importance for us to be able to combat these pathologies. Further, our understanding of daily rhythms can also improve prevention of cardiovascular and cerebrovascular crises (Hastings et. al., 2003). Not only does research and understanding of circadian rhythms provide us with treatments and therapies, but also with the knowledge to prevent illness from occurring in the first place. Understanding how we should schedule and live our daily lives can substantially benefit our well being and quality of life. These findings underline why entrainment, both within individual cells and tissues within an organism, and with the external environment is indeed important.

1.2 - The molecular oscillator

The circadian rhythms in molecular biology, physiology, biochemistry, and behaviour described above are generated by a molecular clock that consists of interlocking transcription/translation feedback loops. The details of this molecular clockwork have been worked out over the past several decades. In animals, the elucidation of the molecular oscillator started when Konopka and Benzer discovered a mutant of a canonical clock gene, now known as *period* (*per*), in *Drosophila* in 1971 (Konopka and Benzer, 1971). Years later, a second gene involved in this oscillator was discovered, *timeless* (*tim*)(Sehgal et al, 1994). In the *Drosophila* molecular oscillator model, during the light phase of a light and dark cycle, also known as photophase, TIM, the product of the *tim* gene, undergoes degradation in the cytoplasm of clock cells (Leloup and Goldbeter, 1998; Hardin, 2009). This degradation is caused by a protein called cryptochrome, which is activated by light. If TIM is degraded, PER, the product of the *per* gene, is unable to form a heterodimer with TIM, and becomes subject to phosphorylation and degradation. These two proteins are phosphorylated in the cytoplasm during the daytime, cannot form a heterodimer, and ultimately cannot translocate into the nucleus. However, during scotophase, TIM degradation by light ceases. This allows TIM to accumulate in the cytoplasm of clock cells, and it is now possible for the PER:TIM heterodimer to form (Hardin, 2009). Heterodimer formation leads to exposure of a nuclear translocation domain, and the PER:TIM complex can now make its way into the nucleus of the clock cell during night-time, where it subsequently interacts with other protein complexes / transcription factors and leads to gene transcription (Bae et. al., 1998). Within the nucleus, there are two more canonical nuclear clock proteins: CLOCK (CLK) and CYCLE (CYC). They were discovered 4 years after *timeless* (Allada et. al., 1998; Price et. al., 1998; Rutila et. al., 1998). By themselves, CLK and CYC bind together, forming a heterodimer (Bae et. al., 1998). They are now capable of starting gene transcription, including *per* and *tim* genes. Once inside the nucleus, the afformentioned PER:TIM complex inhibits CLK:CYC complex function. Therefore, the PER:TIM complex regulates transcription of their own genes via a negative feedback loop (Dunlap, 1996). Making matters even more complicated, there is a second feedback loop within the nucleus. The CLK:CYC complex regulates expression of the *clk* gene (Bae et. al., 1998). The heterodimer complex accomplishes this by initiating transcription of other transcription factor genes, including *vrille (vri)* and *PAR domain protein 1ε (PDP1ε)* (Blau and Young, 1999; Kyriacou et. al., 2008). These, in turn, affect transcription of *clk*, with VRI inhibiting it while PDP1ε promotes it. This feedback loop leads to the CLK:CYC complex being present only at certain times during the day, adding another layer of complexity of the molecular oscillator (Kyriacou et. al., 2008). Overall, the nuclear feedback loop makes CLK:CYC able to form only at certain times, leading to transcription of *per* and *tim* in a timely fashion. Further, because of CRY, the PER:TIM complex formation can only be stable during the night, when it translocates to the nucleus and inhibits transcription of its own genes, by inhibiting the CLK:CYC complex. This constitutes the basis of the molecular oscillator.

The process repeats with a daily rhythm, driving other gene expression and processes in a rhythmic fashion. Thus, cycling PER is a characteristic of clock cells (Dunlap, 1996). It is important to note that PER cycling can be the result of an endogenous molecular oscillator or the product of external cues, for example hormones, driving a slave oscillator within the cell.

It is worthwhile to note that the above described molecular oscillator was described in the insect *Drosophila*, and while the components are highly conserved between fruit flies and mammals (reviewed in Allada et al., 2001; Dunlap, 1999), there are some differences. In mammals, there are three variations of the *period* gene, namely mouse per1 (mper1), mper2, and mper3, as opposed to just one per (dper) in *Drosophila* (Lowrey and Takahashi, 2004; Takahashi et al., 2008). Second, mper forms a heterodimer with CRY instead of TIM (Helfrich-Förster, 2004). Thirdly, within the nucleus, CLK forms a heterodimer with BMAL1 (or NPAS2), the homologue of CYC.

In both the mammalian and *Drosophila* molecular models, the oscillator is able to free run in aperiodic conditions. The exact mechanism by which this happens is currently unknown, but cytoplasmic events such as post-translational modifications of clock proteins might play a role in this process (Kojima et al., 2011; Schmutz et al. 2011).

The distinction between an oscillator and a clock is that a clock needs to be able to be set and entrainable by some form of external Zeitgeber (ie. input), and it needs to have some sort of output, such that it conveys temporal information to other non-clock cells and tissues. On the other hand, a molecular oscillator might not be entrained by external signals, and may not possess a means to provide rhythmic temporal information to other cells and tissues. Further, a molecular oscillator is only part of a clock in cells, as increasing evidence shows that many cytoplasmic components, including post-translational modifications (review by Mehra et al., 2009), are needed and play a crucial role in the cellular time-keeping process (Lakin-Thomas, 2006a, 2006b).

1.2.1 - Circadian organization in mammals – SCN and peripheral clocks

The suprachiasmatic nucleus (SCN), the principal master circadian clock in mammals, is located in the brain, at the base of the hypothalamus, above the optic chiasm (Reppert and Weaver, 2002). The SCN is comprised of two clusters of ~10 000 GABAergic neurons, and is subdivided into two regions: the ventral core, which receives direct innervations from the retina and is responsible for entrainment, and the dorsal shell region, which houses the primary pacemaker cells (Hastings and Herzog, 2004). In aperiodic conditions, rhythms produced by the SCN were found to free run with precision for weeks, even years (Aschoff, 1984; Czeisler and Klerman, 1999). Rhythmic activity of the SCN, including electrical firing rates, metabolism and gene expression persists *in* *vitro* (Aton et. al., 2005; Liu et. al., 2007). Taken together, these findings imply that there is an endogenous clock in the SCN, which is found to be synchronized to the external environment, mainly via light input (Pittendrigh, 1993). Light stimulates a photopigment, melanopsin, located in photoreceptive retinal ganglion cells (Qiu et. al., 2005). Subsequently, this signal is transmitted via direct retinal afferents, stimulating the ventral core region of the SCN, entraining the clock (Hastings et. al., 2007; Hattar et. al., 2003; Panda et. al., 2003). This then leads to the release of gamma-Aminobutyric acid (GABA), vasoactive intestinal peptide (VIP) and gastrin releasing peptide (GRP) (Hastings and Herzog, 2004). These factors act across synapses and activate the dorsal shell region pacemaker neurons, inducing *per* expression. This entrains the PER based molecular clock to the external environment, with light acting as a Zeitgeber (Hastings and Herzog, 2004). The SCN is involved in providing circadian control over a myriad of processes, including: sleep/wakefulness and release of prolactin, cortisol, growth hormone and melatonin secretion during the night time (Czeisler and Klerman, 1999; Hastings et. al., 2007). On top of this, the SCN generates outputs that synchronize transcriptomes, metabolism and physiology of peripheral clocks via endocrine and nervous system routes (Buijs and Kalsbek, 2001; Hastings et. al., 2007).

Neural projections from the SCN relay information into the dorsomedial hypothalamus (Saper et. al., 2005) and from there, extensive networks feed into arousal, sleep regulatory, as well as a diverse range of autonomic nervous centres (Hastings et. al., 2007). This makes it possible for visceral tissue to receive both parasymphatetic and sympathetic innervations. These innervations would convey temporal information in a circadian fashion (Kalsbeek et. al., 2006). This timing information then drives rhythmic processes such as glucocorticoid synthesis and release by the adrenal glands (Ishida et. al., 2005) and melatonin by the pineal gland (Wyatt et. al., 2006). Projections to the paraventricular nucleus, preoptic area, and medio-basal nuclei provide regulation of daily rhythms of adrenocorticotropins, gonadotrophins and metabolic hormones, respectively (Kalsbeek et. al., 2006). Further, it regulates circadian control of secretion of paracrine factors such as growth factor – alpha and neuropeptide cardiotrophin-like cytokines (Kraves and Weitz, 2006). Further, the SCN provides circadian synchronization and control of peripheral tissues, some of which contain their own clockwork (Hastings et. al., 2007). It accomplishes this via nervous connections as previously mentioned above, as well as circulating factors such as hormones (ie. adrenocorticotropic (ACTH), melatonin) (Hastings et. al., 2007). Most major organ systems, such as liver, heart, kidney and skeletal muscle possess endogenous circadian clocks (Balsalobre et. al., 1998; Yamazaki et. al., 2000). While these peripheral clocks maintain circadian rhythms, and have the same molecular oscillator components as the SCN master clock (Nagoshi et. al., 2004), these potential autonomous oscillators across the organism are synchronized by the SCN – making sure all the organs, tissues and cells within an organism work together in synchrony. Thus, the SCN is at the top of the hierarchical circadian axis in mammals, and is responsible for sustaining circadian organization at the level of particular organs and peripheral clocks, as well as synchronizing the organism as a whole to the external environment (Hastings et. al., 2007).

1.2.2 - Circadian organization in insects – Brain and peripheral clocks

In cockroaches, the brain was documented to be involved in the driving of rhythmicity in locomotor activity, as lesioning the area close to the optic lobe in the medulla region lead to the loss of this rhythm (Roberts, 1974; Sokolove, 1975; Page, 1978). This lesion was found to lead to a loss of both locomotor and stridulation activity rhythms in crickets as well (Sokolove and Loher, 1975). The brain clock was further identified in the optic lobe, as transplantation experiments from rhythmic animals to arrhythmic ones, after removal of both optic lobes, restored rhythmicity in host animals (Page, 1982). When this region from the optic lobe from both cockroaches and crickets was placed *in vitro,* self-sustained circadian rhythms in neural activity were found to persist, further showing that this region is indeed housing an endogenous circadian clock (Cowell and Page, 1990; Tomioka and Chiba, 1992).

Documentation of the anatomy of the brain clock in insects was first accomplished in *Drosophila*, and initiated mainly by staining neurons for canonical clock proteins PER, and to a lesser extent, TIM (Liu et. al., 1988; Zerr et. al., 1990; Hunter-Ensor et. al., 1996). Regions of the brain that immunocytologically stained for these proteins included: photoreceptor cells of the compound eyes, ocelli, and some neurons. These neurons were named Lateral Neurons (LNs) and Dorsal Neurons (DNs), depending on their localization in the brain. The next step in elucidating the pacemaker organization within the insect brain employed staining for pigment dispersing factor (PDF), a neuropeptide present in neurons (Helfrich-Förster, 1995), providing a neuromodulator function in controlling circadian rhythms (Park and Hall, 1998; Helfrich-Förster et al, 2000). LNs were found to stain for PDF, and their axons were shown to project into the dorsal central brain, terminating next to the DNs. Further, some LNs were also found to project into the medulla, from where they formed a widespread network with terminal varicosities. Moreover, some LN axons connect both brain hemispheres together, via projections running in the posterior optic tract. Neurons containing the canonical clock proteins PER and TIM were found to have projections in the dorsal protocerebrum, a brain area that connects most sites of the brain, in addition to housing the vast majority of neurosecretory systems in *Drosophila* (Helfrich-Forster, 2003).

This timing system is also entrained by external light (zeitgeber) (reviewed by Foster and Helfrich-Forster, 2001). In *Drosophila* and other insects circadian pacemakers are entrained by several routes including the compound eyes, and the ocelli, as well as by direct penetration of light through the semi-transparent cuticle to act directly on the aforementioned CRY in LNs.

The only other insect in which true brain clock cells have been shown to exist is *Rhodnius* (Vafopoulou et. al., 2010). Similar to the *Drosophila* model, a group of 8 LNs in the proximal optic lobe were identified. Their projections reached the accessory medulla, laterally toward the compound eye and medially into an area of significant arborisation in the anterior protocerebrum. LNs are also found to connect the two brain hemispheres, and also project to the DNs, which are localized in the posterior dorsal protocerebrum. Like the *Drosophila* DNs,*Rhodnius* DNs do not posses PDF, such that their axonal projections are more difficult to map. Nevertheless, this timing system was shown to have the ability to produce rhythmic output, as release of prothoracicotropic hormone (PTTH) was found to be under such control (Vafopoulou and Steel, 1996a, b). This is possible as clock cells in the brain were found to come in close contact and modulate PTTH secreting neurons, in a circadian fashion (Vafopoulou et. al., 2007). Moreover, bombyxin and testis ecdysiotopin peptides (Vafopoulou and Steel, 2002; Vafopoulou and Steel, 2005) were also found to be under circadian control via this brain timing system (Vafopoulou and Steel, 2012). Downstream, these rhythmic outputs could modulate and/or synchronize peripheral clocks. For instance, the prothoracic glands secrete ecdysteroids in a circadian manner, and this is controlled by PTTH in the haemolymph, which entrains the clock in the prothoracic glands (Pelc and Steel, 1997).

Overall, these data show that this brain pacemaker in insects is able to send signals in a circadian fashion to other cells, tissues, and organs in the body through the nervous or humoral system. Further, light input via photoreceptor pigments such as CRY give this timing system the ability to entrain itself to the outside photoperiod, making insects able to synchronize with the external environment, like the SCN in mammals.

1.3 - Fat body of insects

The fat body (FB) of insects is analogous to the mammalian liver and adipose tissue. It has a vast array of metabolic functions, such as the storage and release of energy, lipid metabolism, synthesis and secretion of proteins, and detoxification, all of which are essential for an insect's ability to successfully develop and reproduce (Arrese and Soulages, 2010). In order for the insect to maintain homeostasis and survive, the above processes have to be initiated and regulated at specific times during each day and throughout the life of an insect. Fat body would have to receive some sort of temporal information, such that it would be in synchrony with the rest of the animal, and the external environment. It is known that the central clock in the brain modulates FB function via the endocrine system (DiAngelo et al., 2011; Fallon et. al., 1974). Hormonal control of fat body will be described in more detail in section 1.3.2.

1.3.1 – Localization, morphology, and function

Fat body (FB) of insects is a multifactorial organ which has mesodermal origins (Arrese and Soulages, 2010). It is a relatively large organ, distributed throughout the body of the insect, underneath the integument as well as surrounding the gut, organs, and epidermis (Dean et. al., 1985). FB can be subdivided into two main regions: the subcuticular/peripheral region, located next to the epidermis, and the perivisceral/visceral region that surrounds the gut (Haunerland and Shirk, 1995). There are additional distinct lobes along the anterior-posterior axis (Rizki, 1978). FB is composed of thin, loose lobes of highly tracheated tissue, suspended in the hemocoel and bathed in the haemolymph, having maximal exposure to circulating factors such as nutrients, proteins, and hormones (Haunerland and Shirk, 1995; Lipovsek et. al., 2011). The main cell that constitutes this tissue is the adipocyte (Dean et. al., 1985). Perhaps the most noticeable characteristic of adipocytes are their large vacuoles, which function as a reservoir for lipid storage, mainly triglycerides. Of important note is that there is some variety between species in terms of fat body morphology (Haunerland and Shirk, 1995), and regional localization (Anderson, 1972). Furthermore, there is some evidence that distinct fat body lobes vary somewhat in terms of ultrastructure, biochemistry, synthesis and uptake of storage proteins and even gene expression pattern (Haunerland and Shirk, 1995; Rizki, 1978).

As previously mentioned, the first role of fat body is the ability of adipocytes to store large amounts of energy in the form of lipids (Arrese and Soulages, 2010). It provides the accumulation of nutrients stores for an insect to survive through metamorphosis (Mirth and Riddiford, 2007). Additionally, mobilizing these energy reserves towards the ovaries is necessary during egg development. In some insects, adults don't feed and the fat body has the ability to provide all the energy required for successful egg maturation (Briegel, 1990). This lipid mobilization happens via lipophorins (lipid transporters in the haemolymph). As much as 40% of the fat body weight is lipid and can be lost to the ovaries during egg development (Lorenz and Anand, 2004; Ziegler and Ibrahim, 2001; Ziegler and Van Antwerpen, 2006). This mobilization of lipid stores seems to have a role in immune responses as well, suggesting a yet additional role of fat body in combating infections (Cheon et. al., 2006; Mendes et. al., 2008).

In addition to energy storage, FB is also responsible for metabolic and biosynthetic activities (Arrese and Soulages, 2010). It serves in the metabolism of lipids and carbohydrates and in nitrogen detoxification (Keeley, 1985; Law and Wells, 1989). Moreover, FB is involved in the synthesis of fat, glycogen, and most of the proteins in the haemolymph, including storage proteins such as SP1 and 30k (Kishimoto et. al., 1999), that serve as an amino acid reservoir during metamorphosis and moults (Arrese and Soulages, 2010; Keeley, 1985). Also, it synthesizes vitellogenin, which is essential for egg development and maturation (Aquirre et al., 2008). In *Drosophila*, it was shown that FB also secretes collagen and perlecan, which are incorporated in basement membranes surrounding and protecting organs (Pastor-Pareja and Xu, 2011). Further, in insects such as *Chironomus thummi*(Diptera), FB has a role in respiration, by producing hemoglobin (Bergtrom et. al., 1976; Burmester and Hankeln, 2006). Fat body can also synthesize and secrete metabolites such as acetate and propionate in the haemolymph (Halarnkar and Schooley, 1995). Additionally, FB has been described as an endocrine organ (Ferrandon et. al., 2007), as secreted factors have control over the overall growth of the organism (Colombani et. al., 2003; Hyun, 2013). All these diverse processes and functions of the FB have to be regulated within each daily cycle, as well as during each developmental stage of an insect.

1.3.2 – Hormonal control

Since fat performs multiple metabolic functions to fulfil changing physiological needs of an insect during each day and different developmental stage, fat body requires integrating signals from other organs and the rest of the animal, in order to synchronize all of them together towards a common goal (Gade, 2004). The development and control of function in fat body can be divided into three classes of factors: (1) Ecdysteroids (Ecd) and juvenile hormone (JH); (2) Insulin and adipokinetic hormone (AKH); and (3) Nutrients and stress (Riddiford et. al., 2003). Alteration of Ecd, JH, Insulin and AKH can lead to developmental defects at the organism level (Britton et. al., 2002; Colombani et.

al., 2005). Non-optimal levels of circulating hormones such as ecdysone and insulins can lead to drastic changes in the size of the insect, including death (Colombani et. al., 2005).

Ecd and JH are key developmental hormones in insects, and both have important roles in regulating FB (Liu et. al., 2009). Ecd were shown to play a vital role in protein synthesis and secretion by the FB in the silkworm *Bombyx mori* (Rajathi et. al., 2010). Manipulating Ecd levels was found to lead to abnormal development, metamorphosis, and haemolymph proteins, including vitellogenin. In the mosquito *Aedes aegypti,* fat body was found to synthesize vitellogenin after a blood meal (Hagedorn, 1973), a process induced by circulating Ecd (Fallon et. al., 1974). Moreover, Ecd levels were also found to correlate with the amount of vitellogenin produced in the housefly *Musca domestica* (Adams et. al., 1997). Moreover, Ecd was found to lead to energy mobilization by the fat body (Liu et. al., 2009). JH was also shown to contribute to vitellogenin synthesis and secretion by the fat body, by activating transcription of certain genes (Glinka and Wyatt, 1996; Jensen and Brasch, 1985). Also, JH is involved in the nuclear development of adipocytes. Deficiencies in JH were found to lead to FB remodelling (Liu et. al., 2009).

AKH activates the enzymes glycogen phosphorylase and lipase in the fat body, leading to increased haemolymph carbohydrates, lipids, and proline concentrations (reviewed by Gade, 2004). This mobilization of energy has a role in providing flight muscles with much needed energy in flying insects. Moreover, AKH can provide an inhibitory signal to the fat body, decreasing synthesis of RNA, fatty acids, and proteins (Lorenz and Anand, 2004). Insulin-like peptides have also been found to have a regulatory role in the metabolism and growth of FB (Liu et. al., 2009). In *Bombyx* mori, it was shown that a reduced insulin signal (ie. lower levels of insulin) works synergistically with an increased Ecd signal in energy mobilization.

In addition to hormones, nutrient levels and stress also have a regulatory role on FB. For example, nutrient deprivation and stress were found to lead to activation of protein kinases, which subsequently regulate FB metabolism and transcription of certain genes (Okamura et. al., 2007). Overall, FB is a central organ to integrate hormonal and nutritional signals in order to regulate insect development and metamorphosis and reproduction (Colombani et. al., 2005). Additionally, hormonal input could provide temporal information to fat body, a potential peripheral clock, or slave oscillator.

1.4 - The study of circadian rhythms using fat body of Rhodnius prolixus (Hemiptera) as a model organism

Rhodnius has proved to be a good model organism for studying the circadian control of fat body for three reasons. First, unfed *Rhodnius* larvae exist in a state of developmental arrest. A blood meal triggers the start of development from one larval stage to the next **(**Buxton, 1930). As such, it is extremely easy to synchronize development within a population of animals. Second, insects in the 5th larval stage are large enough for dissections to be carried out. Further, it was easy to isolate a distinct fat body lobe for dissection at each time point – the one next to the prothoracic glands. This was done such that examination of fat body is done looking at the same lobe and adipocytes at each time point. This was important, as distinct fat body lobes can exhibit regional differentiation with respect to ultrastructure, metabolism and gene expression (see 1.3.1). Third, fat body of *Rhodnius* was already known to express PERIOD (unpublished results from our lab), the protein I looked at for examining circadian activity in this organ.

1.5 - Objectives

Is there temporal regulation of fat body cells? This study set out to elucidate the circadian control of FB, mainly, whether fat body possesses an endogenous clock or molecular oscillator, or whether temporal control is achieved via circulating hormones. In order to do so, the following objectives were formulated: (1) to determine if PER cycles with a daily rhythm in FB cells; (2) to examine whether PER cycling is under endogenous circadian clock control (i.e. persists in aperiodic conditions *in vivo*); (3) to examine whether circadian control of PER cycling is endogenous to the fat body, or not (i.e. PER rhythm persists *in vitro*), and; (4) if not, how is PER cycling in the FB controlled?

1.6 - References

Adams TS, Gerst JW, Masler EP. 1997. Regulation of ovarian ecdysteroid production in the housefly, *Musca domestica*. *Arch Insect Biochem***35:**135-148.

Allada R, Emery,P, Takahashi JS, Rosbash M. 2001. Stopping time: the genetics of fly and mouse circadian clocks. *Annu Rev Neurosci***24:**1091–1119.

Allada R, White NE, So WV, Hall JC, Rosbash M. 1998. A mutant *Drosophila* homolog of mammalian Clock disrupts circadian rhythms and transcription of *period* and *timeless*. *Cell***93:**791-804.

Anderson DT. 1972. The development of holometabolous insects. In: Counce SJ, Waddington CH (eds) *Developmental Systems: Insects*. New York: Academic Press.

Aquirre SA, Frede S, Rubiolo ER, Canavoso LE. 2008. Vitellogenesis in the hematophagous *Dipetalogaster maxima* (Hemiptera: Reduviidae), a vector of Chagas' disease. *J Insect Physiol***54:**393-402.

Arrese EL, Soulages JL. 2010. Insect fat body: energy, metabolism and regulation. *Annu Rev Entomol***55:**207-225.

Aschoff J. 1984. Circadian timing. *Ann NY Acad Sci***423:**442–468.

Aton SJ, Colwell CS, Harmar AJ, Waschek J, Herzog ED. 2005. Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. *Nature Neurosci***8:**476–483.

Bae K, Lee C, Sidnote D, Chuang K, Edery I. 1998. Circadian regulation of a *Drosophila* homolog of the mammalian clock gene: PER and TIM function as positive regulators. *Mol Cell Biol***18(10):**6142-6151.

Balsalobre A, Damiola F, Schibler U. 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell***93:**929–937.

Barclay J, Husse J, Oster H. 2011. Adrenal glucocorticoids as a target for jet lag therapies. *Expert Rev Endocrinol Metab***6(5):**673-685.

Bell-Pedersen D, de Paula RM, Vitalini MV. 2006. The rhythms of life: circadian output pathways in *Neurospora. J Biol Rhythms***21:**432-444.

Bergtrom G, Laufer H, Rogers R. 1976. Fat body: a site of hemoglobin synthesis in *Chironomus thummi* (Diptera). *J Cell Biol* **69:**264–274.
Blau J, Young MW. 1999. Cycling *vrille* expression is required for a functional *Drosophila* clock. *Cell* **99(6)**:661-671.

Briegel H. 1990. Metabolic relationship between female body size, reserves, and fecundity of *Aedes aegypti*. *J Insect Physiol* **36:**165–72.

Britton JS, Lockwood WK, Li L, Cohen SM, Edgar BA. 2002. *Drosophila*'s insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. *Dev Cell* **2:**239–249.

Bünning E. 1936. Die endogene Tagesrhythmik als Grundlage der photoperiodischen Reaktion. *Ber Dtsch Bot Ges* **54:**590–607.

Buijs RM, Kalsbeek A. 2001. Hypothalamic integration of central and peripheral clocks. *Nature Neurosci* **2:**521–526.

Burmester T, Hankeln T. 2006. The respiratory proteins of insects. *J Insect Physiol* **53:**285-294.

Buxton PA. 1930. The biology of a blood sucking bug, *Rhodnius prolixus*. *T Roy Entomol Soc Lond* **78(2):**227-256.

Castrejón-Gómez VR. 2010. Evidence of a sex pheromone and daily calling pattern of females of *Zamagiria dixolophella* (Lepidoptera: Pyralidae). *Fla Entomol* **93(2):**147-152.

Cauter TV, Poucet B, Save E. 2008. Delay-dependent involvement of the rat entorhinal cortex in habituation to a novel environment. *Neurobiol Learn Mem* **90:**192-199.

Cheon HM, Shin SW, Bian G, Park JH, Raikhel AS. 2006. Regulation of lipid metabolism genes, lipid carrier protein lipophorin, and its receptor during immune challenge in the mosquito *Aedes aegypti*. *J Biol Chem* **281:**8426-8435.

Colombani J, Bianchini L, Layalle S, Pondeville E, Dauphin-Villemant C, Antoniewski C, Carre C, Noselli S, Leopold P. 2005. Antagonistic actions of ecdysone and insulin determine final size in Drosophila. *Science* **310:**667–670.

Colombani J, Raisin S, Pantalacci S, Radimerski T, Montagne J, Leopold P. 2003. A nutrient sensor mechanism controls *Drosophila* growth. *Cell* **114(6)**:739–749.

Cowell CS, Page TL. 1990. A circadian rhythm in neural activity can be recorded from the central nervous system of the cockroach. *J Comp Physiol A* **166(5)**:643-649.

Czeisler CA, Klerman EB. 1999. Circadian and sleep-dependent regulation of hormone release in humans. *Recent Prog Horm Res***54:**97-132.

Dallaspezia S, Benedetti F. 2009. Melatonin, circadian rhythms, and the clock genes in bipolar disorder. *Curr Psychiatry Rep***11:**488-493.

Davidson AJ, Straume M, Block GD, Menaker M. 2006. Daily timed meals dissociate circadian rhythms in hepatoma and healthy host liver. *Int J Cancer***118:**1623-1627.

Dean RL, Collins JV, Locke M. 1985. Structure of the fat body. In: Kerkut GA, Gilbert LI (eds) *Comprehensive Insect Physiology, Biochemistry, and Pharmacology.* pp. 155–210. New York: Pergamon.

deCandolle MAP. 1832. Bechet Jeune Library Faculty Medicine. *Physiologie vegetale***111**.

deMairan JJD. 1729. Observation Botanique. Paris: *Hist Acad R Sci***35**.

DiAngelo JR, Erion R, Crocker A, Sehgal A. 2011. The central clock neurons regulate lipid storage in *Drosophila*. *PLoS one***6(5):**e19921.

Dunlap J. 1999. Molecular bases for circadian clocks. *Cell***96:**271–290.

Dunlap JC, Loros JJ. 2006. How fungi keep time: circadian system in *Neurospora* and other fungi. *Curr Opin Microbiol***9:**579-587.

Dunlap JC. 1996. Genetic and molecular analysis of circadian rhythms. *Annu Rev Gen***30:**579-601.

Eckel-Mahan KL. 2012. Circadian oscillations within the hippocampus support memory formation and persistence. *Front Mol Neurosci***5:**1-4.

Fallon AM, Hagedorn HH, Wyatt GR, Laufer H. 1974. Activation of vitellogenin synthesis in the mosquito *Aedes aegypti* by ecdysone. *J Insect Physiol***20(9):**1815-1823.

Ferrandon D, Imler JL, Hetru C, Hoffmann JA. 2007. The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. *Nat Rev Immunol* **7:**862–874.

Filipski E, Delaunay F, King VM, Wu MW, Claustrat B, Grechez-Cassiau A, Guettier C, Hastings MH, Francis L. 2004. Effects of chronic jet lag on tumor progression in mice. *Cancer Res***64:**7879-7885.

Filipski E, King VM, Li X, Granda TG, Mormont MC, Liu X, Claustrat B, Hastings MH, Levi F. 2002. Host circadian clock as a control point in tumor progression. *J Nat Cancer I***94:**690-697.

Foster RG, Helfrich-Förster C. 2001. The regulation of circadian clocks by light in fruitflies and mice. *Phil Trans R Soc Lond B***356**:1779-1789.

Gade G. 2004. Regulation of intermediary metabolism and water balance of insects by neuropeptides. *Annu Rev Entomol***49:**93-113.

Germain A, Kupfer DJ. 2008. Circadian rhythm disturbances in depression. *Hum Psychopharmacol Clin***23:**571-585.

Glinka AV, Wyatt GR. 1996. Juvenile hormone activation of gene transcription in locust fat body. *Insect Biochem Mol Biol***26:**13–18.

Green RM, Tingay S, Wang ZY, Tobin EM. 2002. Circadian rhythms confer a higher level of fitness to Arabidopsis plants. *Plant Physiol***129:**576-584.

Hagedorn HH, Fallon AM, Laufer H. 1973. Vitellogenin synthesis by the fat body of the mosquito *Aedes aegypti*: evidence for transcriptional control. *Dev Biol***31(2):**285-294.

Halarnkar PP, Schooley DA. 1995. A comparative catabolism study of isoleucine by insect and mammalian tissues. *Comp Biochem Physiol***110(2):**357-365.

Hardin PE. 2009. Molecular mechanisms of circadian timekeeping in *Drosophila*. *Sleep Biol Rhythm***7(4):**235-242.

Hastings MH, Herzog ED. 2004. Clock genes, oscillators, and cellular networks in the suprachiasmatic nuclei. *J Biol Rhythm***19:**400–413.

Hastings MH, O'Neill JS, Maywood, ES. 2007. Circadian clocks: regulators of endocrine and metabolic rhythms. *J Endocrinol***195:**187-198.

Hastings MH, Reddy AB, Maywood ES. 2003. A clockwork web: circadian timing in brain and periphery, in health and disease. *Nat Neurosci***4:**649-661.

Hattar S, Lucas RJ, Mrosovsky N, Thompson S, Douglas RH, Hankins MW, Lem J, Biel M, Hofmann F, Foster RG, Yau KW. 2003. Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. *Nature***424:**76–81.

Haunerland NH, Shirk PD. 1995. Regional and functional differentiation in the insect fat body. *Annu Rev Entomol***40:**121-145.

Helfrich-Förster C. 1995. The *period* clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. P*roc Natl Acad Sci USA***92:**612-616.

Helfrich-Förster C. 2003. The neuroarchitecture of the circadian clock in the brain of *Drosophila melanogaster*. *Microsc Res Tech***62:**94-102.

Helfrich-Förster C. 2004. The circadian clock in the brain: a structural and functional comparison between mammals and insects. *J Comp Physiol A***190:**601-613.

Helfrich-Förster C, Tauber M, Park JH, Muhlig-Versen M, Schneuwly S, Hofbauer A. 2000. Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. *J Neurosci***20:**3339-3353.

Hunter-Ensor M, Ousley A, Sehgal A. 1996. Regulation of the *Drosophila* protein TIMELESS suggests a mechanism for resetting the circadian clock by light. *Cell***84:**677-685.

Hyun S. 2013. Body size regulation and insulin-like growth factor signaling. *Cell Mol Life Sci***70(13):**2351-2365.

Ishida A, Mutoh T, Ueyama T, Bando H, Masubuchi S, Nalahara D, Tsujimoto G, Okamura H. 2005. Light activates the adrenal gland: timing of gene expression and glucocorticoid release. *Cell Met***2:**297-207.

Jensen Al, Brasch K. 1985. Nuclear development in locust fat body: the influence of juvenile hormone on inclusion bodies and the nuclear matrix. *Tissue Cell***17(1):**117-130.

Johnson CH, Knight M, Trewavas A, Kondo T. 1998.A clockwork green: circadian programs in photosynthetic organisms. In: Lumsden PJ, Millar AJ (eds)*Biological rhythms and photoperiodism in plants*. pp. 1-34. Oxford: Bios.

Kalsbeek A, Palm IF, La Fleur SE, Scheer FA, Perreau-Lenz S, Ruiter M, Kreier F, Cailotto C, Buijs RM. 2006. SCN outputs and the hypothalamic balance of life. *J Biol Rhythm***21:**458–469.

Keeley LL. 1985. Biochemistry and physiology of the insect fat body. In: Kerkut GA, Gilbert LI (eds)*Comprehensive Insect Physiology, Biochemistry and Pharmacology*. pp. 211–228. New York: Pergamon.

Kishimoto A, Nakato H, Izumi S, Tomino S. 1999. Biosynthesis of major plasma proteins in the primary culture of fat body cells from the silkworm, *Bombyx mori*. *Cell Tissue Res***297:**329-335.

Klarsfeld A, Rouyer F. 1998. Effects of circadian mutations and LD periodicity on the life span of *Drosophila melanogaster*. *J Biol Rhythm* **13:**471-478.

Knutsson A. 2003. Health disorders of shift workers. *Occup Med***53:**103-108.

Kondo T, Ishiura M. 2000. The circadian clock of cyanobacteria. *BioEssays***22:**10-15.

Konopka RJ, Benzer S. 1971. Clock mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci USA***68(9):**2112-2116.

Kraves S, Weitz CJ. 2006. A role for cardiotrophin-like cytokine in the circadian control of mammalian locomotor activity. *Nat Neurosci***9:**212–219.

Kyriacou CP, Peixoto AA, Sandrelli F, Costa R, Tauber E. 2008. Clines in clock genes: fine-tuning circadian rhythms to the environment. *Trends Genet***24:**124-132.

Lakin-Thomas P. 2006a. New models for circadian systems in microorganisms. *FEMS Microbiol Lett***259**:1–6.

Lakin-Thomas P. 2006b. Transcriptional feedback oscillators: Maybe, maybe not… *J Biol Rhythm***21**:83–92.

Law JH,Wells MA. 1989. Insects as biochemical models. *J Biol Chem* **2641:**6335–38.

Leloup J, Goldbeter A. 1998. A model for circadian rhythms in *Drosophila* incorporating the formation of a complex between the PER and TIM proteins. *J Biol Rhythm***13(1):**70-87.

Levi F, Schibler U. 2007. Circadian rhythms: mechanisms and therapeutic implications. *Annu Rev Pharmacol Toxicol***47:**593-628.

Lipovsek S, Novak T, Janzekovic F, Pabst MA. 2011. Role of the fat body in the cave crickets *Troglophilus cavicola* and *Troglophilus neglectus* (Rhaphidophoridae, Saltatoria) during overwintering. *Arthropod Struct Dev***40:**54-63.

Liu AC,Welsh DK, Ko CH, Tran HG, Zhang EE, Priest AA, Buhr ED, Singer O, Meeker K, Verma IM, Doyle FJ 3rd, Takahashi JS, Kay SA. 2007. Intercellular coupling confers robustness against mutations in the SCN circadian clock network. *Cell***129:**605–616.

Liu X, Lorenz L, Yu Q, Hall JC, Rosbash M. 1988. Spatial and temporal expression of the *period* gene in *Drosophila melanogaster*. *Genes Dev* **2:**228-238.

Liu Y, Liu H, Liu S, Wang S, Jiang RJ, Li S. 2009. Hormonal and nutritional regulation of insect fat body development and function. *Arch Insect Biochem Physiol* **74(1):**16-30.

Lorenz MW, Anand AN. 2004. Changes in the biochemical composition of fat body stores during adult development of female crickets, *Gryllus bimaculatus*. *Arch Insect Biochem Physiol* **56:**110–119.

Lowrey PL, Takahashi JS. 2004. Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annu Rev Genomics Hum Genet* **5:**407-441.

Mehra A, Baker CL, Loros JJ, Dunlap JC. 2009. Post-translational modifications in circadian rhythms. *Trends Biochem Sci* **34(1):**483-490.

Mendes AM, Schlegelmilch T, Cohuet A, Awono-Ambene P, De Iorio M, Fontenille D, Morlais I, Christophides GK, Kafatos FC, Vlachou D. 2008. Conserved mosquito/parasite interactions affect development of *Plasmodium falciparum* in Africa. *PLoS Pathog* **4:**e1000069.

Mirth CK, Riddiford LM. 2007. Size assessment and growth control: how adult size is determined in insects. *BioEssays* **29:**344–55.

Nagoshi E, Saini C, Bauer C, Laroche T, Naef F, Schibler U. 2004. Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* **119:**693–705.

Oishi K, Amagai N, Shirai H, Kadota K, Ohkura N, Ishida N. 2005. Genome-wide expression analysis reveals 100 adrenal gland-dependent circadian genes in the mouse liver. *DNA Res*. **12:**191-202.

Oishi M, Suwazini Y, Sakata K, Okubo Y, Harada H, Kobayashi E, Uetani M, Nogawa K. 2005. A longitudinal study on the relationship between shift work and the progression of hypertension in male Japanese workers. *J Hypertens* **23:**2173-2178.

Okamura T, Shimizu H, Nagao T, Ueda R, Shunsuke I. 2007. ATF-2 regulates fat metabolism in *Drosophila*. *Mol Biol Cell* **18:**1519-1529.

Page TL. 1978. Interaction between bilaterally paired components of the cockroach circadian system. *J Comp Physiol* **124:**225-236.

Page TL. 1982. Transplantation of the cockroach circadian pacemaker. *Science* **216:** 73-75.

Panda S, Provencio I, Tu DC, Pires SS, Rollag MD, Castrucci AM, Pletcher MT, Sato TK, Wiltshire T, Andahazy M, Kay SA, Van Gelder RN, Hogenesch JB. 2003. Melanopsin is required for nonimage-forming photic responses in blind mice. *Science* **301:** 525–527.

Park JH, Hall JC. 1998. Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in *Drosophila melanogaster*. *J Biol Rhythms* **13:** 219-228.

Park JH, Schroeder AJ, Helfrich-Förster C, Jackson FR, Ewer J. 2003. Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and circadian timing of ecdysis behaviour. *Dev* **130:** 2645-2656.

Pastor-Pareja J, Xu T. 2011. Shaping cells and organs in *Drosophila* by opposing roles of fat body-secreted collagen IV and perlecan. *Dev Cell* **21:** 245-256.

Pelc D, Steel CGH. 1997. Rhythmic steroidogenesis by the prothoracic glands of the insect *Rhodnius prolixus* in the absence of rhythmic neuropeptide input: implications for the role of prothoracicotropic hormone. *Gen Comp Endocrinol* **108(3):** 358-365.

Pittendrigh CS. 1954. On temperature independence in the clock system controlling emergence in *Drosophila*. *Proc Nat Acad Sci USA* **40:** 1018-29.

Pittendrigh CS. 1993. Temporal organization: reflections of a Darwinian clock-watcher. *Annu Rev Physiol* **55:** 17-54.

Pittendrigh CS, Bruce, VG. 1957. An oscillator model for biological clocks. In: Rudnik D (ed) *Rhythmic and Synthetic Processes in Growth*, pp. 75-109. Princeton: Princeton University Press.

Price JL, Blau J, Rothenfluh A, Abodeely M, Loss B, Young MW. 1998. Double-time is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell* **94:** 83-95.

Qiu X, Kumbalasiri T, Carlson SM, Wong KY, Krishna V, Provencio I, Berson DM. 2005. Induction of photosensitivity by heterologous expression of melanopsin. *Nature* **433:** 745–749.

Rajathi A, Pandiarajan J, Krishnan M. 2010. Effect of RH-2485 on development, metamorphosis and synthesis of major proteins in female silkworm *Bombyx mori*. *Biologia***65(5):**903-913.

Reppert SM, Weaver DR. 2002. Coordination of circadian timing in mammals. *Nature***418:**935–941.

Riddiford LM, Hiruma K, Zhou X, Nelson CA. 2003. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. *Insect Biochem Mol Biol***33:**1327–1338.

Rizki TM. 1978. Fat body. In: Ashburner M, Wright TRF (eds)*The Genetics and Biology of Drosophila*, pp. 561—601. London: Academic.

Roberts SK. 1974. Circadian rhythms in cockroaches: effects of optic lobe lesions. *J Comp Physiol***88:**21-30.

Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC. 1998. CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drorophila period* and *timeless*. *Cell***93:**805-814.

Salgado-Delgado R, Osorio AT, Saderi N, Escobar C. 2011. Disruption of circadian rhythms: a crucial factor in the etiology of depression. *Depress Res Treat***2011:**1-9.

Saper CB, Scammell TE, Lu J. 2005. Hypothalamic regulation of sleep and circadian rhythms. *Nature***437:**1257–1263.

Schernhammer ES, Kroenke CH, Laden F, Hankinson SE. 2006. Night work and risk of breast cancer. *Epidemiol***17:**108-111.

Schernhammer ES, Laden F, Speizer FE, Willett WC, Hunter DJ, Kawachi I, Fuchs CS, Colditz GA. 2003. Night-shift work and risk of colorectal cancer in the nurses' health study. *J Nat Cancer I***95:**825-828.

Sehgal A, Price JL, Man B, Young MW. 1994. Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant timeless. *Science***263:**1603-1606.

Sokolove PG, Loher W. 1975. Role of eyes, optic lobes and and pars intercerebralis in locomotory and stridulatory circadian rhythm of *Teleugryh commodus*. *J Insect Physiol***21:**785-799.

Sokolove PG. 1975. Localization of the cockroach optic lobe circadian pacemaker with microlesions. *Brain Res***87:**13-21.

Sookoian S, Gemma C, Fernandez GT, Burgueno A, Alvarez A, Gonzalez CD, Pirola CJ. 2007. Effects of rotating shift work on biomarkers of metabolic syndrome and inflammation. *J Intern Med***261:**285-292.

Takahashi JS, Hong HK, Ko CH, McDearmon EL. 2008. The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet***9:**764-775.

Tomioka K, Chiba Y. 1992. Characterization of optic lobe circadian pacemaker by *in situ* and *in vitro* recording of neural activity in the cricket *Gryllus bimaculatus*. *J Comp Physiol A***171:**1-7.

Vafopoulou X, Steel CGH. 1996a. The insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: re-evaluation of its role in development. *Proc Nat Acad Sci USA***93(8):**3368-3372.

Vafopoulou X, Steel CGH. 1996b. Circadian regulation of a daily rhythm of release of prothoracicotropic hormone from the brain-retrocerebral complex of *Rhodnius prolixus* (Hemiptera) during larval-adult development. *Gen Comp Endocrinol***102(1):**123-129.

Vafopoulou X, Steel CGH. 2002. Prothoracicotropic hormone of *Rhodnius prolixus*: partial characterization and rhythmic release of neuropeptides related to *Bombyx* PTTH and bombyxin. *Inver Rep Dev***42(2):**111-120.

Vafopoulou X, Steel CGH. 2005. Circadian organization of the endocrine system. In: Gilbert LI, IA Trrou K, Gill SS (eds)*Comprehensive Molecular Insect Science*, pp. 551-604. Oxford, Elsevier.

Vafopoulou X, Steel CGH. 2012. Insulin-like and testis ecdysiotropin neuropeptides are regulated by the circadian timing system in the brain during larval-adult development in the insect *Rhodnius prolixus* (Hemiptera). *Gen Comp Endocrinol***179(2):**277-288.

Vafopoulou X, Steel CGH, Terry KL. 2007. Neuroanatomical relations of prothoracicotropic hormone neurons with the circadian timekeeping system in the brain of larval and adult *Rhodnius prolixus* (Hemiptera). *J Comp Neurol***503(4):**511-524.

Vafopoulou X, Terry KL, Steel CGH. 2010. The circadian timing system in the brain of the fifth larval instar of *Rhodnius prolixus* (Hemiptera). *J Comp Neurol***518:**1264-1282.

Webb AAR. 2003. The physiology of circadian rhythms in plants. *New Phytol***160:**281-303.

Woelfle MA, Ouyang Y, Phanvijhitsiri K, Johnson CH. 2004. The adaptive value of circadian clocks; an experimental assessment in cyanobacteria. *Curr Biol* **14:**1481–1486.

Wyatt JK, Dijk DJ, Ritz-de Cecco A, Ronda JM, Czeisler CA. 2006. Sleep facilitating effect of exogenous melatonin in healthy young men and women is circadian-phase dependent. *Sleep* **29:**609–618.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, Tei H. 2000. Resetting central and peripheral circadian oscillators in transgenic rats. *Science* **288:**682–685.

Zerr DM, Hall JC, Rosbash M, Siwicki KK. 1990. Circadian fluctuations of PERIOD protein immunoreactivity in the CNS and the visual system of *Drosophila*. *J Neurosci* **10:**2749-2762.

Ziegler R, Ibrahim MM. 2001. Formation of lipid reserves in fat body and eggs of the yellow fever mosquito, *Aedes aegypti*. *J Insect Physiol* **47:**623–27.

Ziegler R, van Antwerpen R. 2006. Lipid uptake by insect oocytes. *Insect Biochem Mol Biol* **36:**264–72.

CHAPTER II: MANUSCRIPT

A Steroid Driven Peripheral Oscillator in Fat Body Cells of *Rhodnius prolixus* (Hemiptera)

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Running Title: Steroid rhythm drives PER cycling in an insect

ABSTRACT

The insect fat body (FB) performs functions as diverse as the mammalian liver, with which it is functionally analogous. These functions must be coordinated with those of other organs and with the external world. We examined this coordination in fifth instar larvae of *Rhodnius prolixus* in FB, using confocal microscope immunohistochemistry and Western blots for the canonical clock protein, PERIOD (PER). PER fluorescence was found in both nuclei and cytoplasm of FB cells *in vivo*. Nuclear and cytoplasmic PER fluorescence exhibited cycles in abundance that free ran in continuous darkness (DD) *in vivo*. PER was severely depleted in prolonged continuous light (LL). On transfer to DD PER was induced and then cycled with circadian periodicity in both nuclei and cytoplasm *in vivo*. When fat body cells from LD were incubated *in vitro* in LD, cycling of PER ceased. We infer that cycling of PER in fat body cells is not indicative of an autonomous circadian clock in this tissue. Addition of 20-hydroxyecdysone (molting hormone) to fat body cells *in vitro* induced PER expression within 1 hour. It is known that *invivo*, 20-hydroxyecdysone levels have a clear circadian rhythm that peaks in the scotophase. Thus, 20-hydroxyecdysone acts synergistically with darkness *in vivo*. These data show that the FB of *Rhodnius* does not constitute an autonomous clock, but rather represents an oscillator that is driven by the circadian rhythm in steroid levels.

Keywords: fat body, circadian rhythm, PER, clock, ecdysteroids, endocrine rhythm, slave oscillator.

1. INTRODUCTION

Circadian rhythms are central to the physiology of organisms. These roughly 24h rhythms are generated by clock cells in which interconnected transcription/translation feedback loops form a molecular oscillator (reviewed by Hardin, 2009). The elucidation of the detailed workings of the molecular clockwork has allowed the recent demonstration of clocks in cells and tissues throughout organisms (Balsalobre et. al., 1998; Yamazaki et. al., 2000; Pelc and Steel, 1997; Vafopoulou and Steel, 1998). These ‘peripheral’ clocks in animals show a range of autonomy from the ‘central’ clock in the brain. Central and peripheral clocks are synchronized into a single coordinated circadian system via nervous and hormonal communication pathways (Buijs and Kalsbek, 2001; Hastings et. al., 2007). The exact mechanisms by which such coordination is achieved remain poorly understood and the relative contribution of central and peripheral clocks to the proper functioning of specific cells and tissues is largely unknown. However, the current view holds that peripheral clocks coordinate local events, but are synchronized throughout the animal by the “master clock” in the brain (Hastings et al., 2007).

Insect fat body is functionally analogous to the mammalian liver and is involved in numerous metabolic activities including lipid, carbohydrate, protein synthesis and storage and detoxification (reviewed by Arrese and Soulages, 2010). The need to coordinate the numerous complex functions of the fat body in a timely fashion leads to the expectation that these functions will be under circadian control. This circadian control may be provided by a local clock in the fat body tissue itself or by clocks outside the fat body that send rhythmic inputs to this tissue. Insect fat body is not innervated and its functions are therefore regulated exclusively by circulating factors in the hemolymph such as hormones. Fat body function is known to be modulated by adipokinetic hormone (AKH), ecdysteroids, insulin-like peptides (ILPs), and juvenile hormone (JH) (Riddiford et. al., 2003; Britton et. al., 2002; Colombani et. al., 2005; Liu et. al., 2009). AKH activates fat body enzymes to mobilize energy stores during increased activity. Ecdysteroids, the insect molting hormones produced by the larval prothoracic glands (PGs), have been shown to modulate protein synthesis and secretion by the fat body(Rajathi et. al., 2010). The brain neuropeptides, ILPs, have also been shown to regulate the growth and metabolism of fat body and to interact with ecdysteroid signaling with respect to energy mobilization by the fat body (Liu et. al., 2009). In adult, JH is synthesized by the corpus allatum(CA) and is a well known regulator of vitellogenin synthesis by fat body during egg development in insects, including *Rhodnius prolixus*(Glinka and Wyatt, 1996; Jensen and Brasch, 1985). The many diverse functions of the fat body are regulated by multiple hormonal signaling pathways, emphasizing the importance and complexity of fat body processes.

The majority of these hormones have been shown to be under circadian control. In *R. prolixus* a circadian rhythm of hemolymph ecdysteroid titer (Ampleford and Steel, 1986) is regulated by a photosensitive clock in the PGs (Vafopoulou and Steel, 1998). The levels of ILPs in the hemolymph of larval and adult *R. prolixus* have recently been shown to fluctuate with a circadian rhythm that is under the control of the brain clock (Vafopoulou and Steel, 2012). JH levels in the hemolymph have been shown to be under circadian control in the cricket *Gryllus bicamulatus* (Zhao and Zera, 2004). Therefore, it seems reasonable to expect that fat body function may be regulated in a circadian manner by rhythmic hormonal inputs. Recently, a local clock within the fat body was described in *Drosophila melanogaster* raising the possibility of more local temporal control of fat body processes in insects (Xu et. al., 2008, 2011).

The classic model organism of insect physiology, *R. prolixus*, is particularly well-suited to circadian investigations. *R. prolixus* remains in a state of developmental arrest until a blood meal triggers development from one larval stage to the next, thereby allowing the precise induction of synchronous development of an entire experimental population.This lab has successfully exploited this insect to elucidate the circadian organization and regulation of larval development (reviewed by Steel and Vafopoulou, 2006). The multi-oscillator circadian system of *R. prolixus* consists of light sensitive clocks in the brain and PGs that interact via rhythmic hormonal signals to ultimately generate the circadian rhythm of ecdysteroid levels in the hemolymph and orchestrate larval development around the 24 h day.

In order to determine circadian regulation in fat body of larval *Rhodnius prolixus*, we employed immunohistochemistry and laser confocal microscopy to study the presence and cycling of the canonical clock protein PERIOD (PER). We report that PER is present in both the cytoplasm and nuclei of fat body cells of *R. prolixus*, and exhibits circadian cycling *in vivo*. PER-IR was undetectable and no longer cycled 7 hours post-dissection *in vitro*. A brief 1 h pulse of 20-hydroxyecdysone (20E) 7 hours after *in vitro* incubation induced rapid and significant PER expression, but PER staining intensity decreased abruptly after another 6 h *in vitro* and eventually disappeared completely; cycling was not observed. We infer that *R. prolixus* fat body represents a driven oscillator and that PER cycling *in vivo* is induced by the known rhythm of ecdysteroid levels in the hemolymph.

2. MATERIALS AND METHODS

2.1 - Animals and Tissues

Rhodnius prolixus were reared in a 12 hours light : 12 hours dark regime (LD) at a constant temperature of 28 ± 0.5^{o}C. Fifth instar larvae were fed a blood meal 1 hour before lights off; the day of feeding is referred to as day 0. Feeding of larvae initiates synchronous development to the next stage; ecdysis to the adult occurs in a gated fashion around day 21 (Ampleford and Steel, 1982). Arrhythmic animals were produced by maintaining animals in continuous light (LL) at 28 ± 0.5^{o}C for a minimum of 4 weeks prior to feeding. This has been shown to abolish both behavioural and hormonal rhythmicity (Vafopoulou and Steel 1991, 1992) and eliminated expression of PER (see Section 3). The FB outer lobe adjacent to the prothoracic glands (PGs), not containing the PGs, of fifth instar males was dissected every 6 hours (at 1h and 7h after lights on and 1h and 7h after lights off) on days 12 and 13 after a blood meal. Insects in darkness remained so until immediately prior to dissection.

2.2 – Antibodies

A custom made (Genscript, Piscataway, NJ) Protein A purified polyclonal antibody produced in guinea pig and raised against a synthetic peptide corresponding to the highly conserved PAS region of the *Drosophila* PERIOD protein (residues 605-618; KSSTETPPSYNQLN; known as peptide PER-S) was employed in order to visualize PER in FB cells. It was used at a dilution of 1:500. This 14-amino acid sequence (PER-S) was found to be highly conserved among different insects, with a 100% sequence identity between *Drosophila melanogaster* and other drosophilids, and with *Anastrepha suspensus*, *Apis mellifera*, *Blatella germanica*, *Ceratitis capita* and *Sargophaga bullata* (Vafopoulou et al., 2010). A 92% identity was found in *Anopheles gambiae*, *Anthereae pernyi*,*Bombyx mori and Musca domestica*,with only 1 amino acid being substituted. Additionally, SDS-PAGE and western blot analysis as well as immunohistochemistry presented here confirm the specificity of this antibody (see Results). Thus, it is highly likely that this antibody recognizes the native PER of *Rhodnius prolixus*.

For immunohistochemistry, anti-guinea pig IgG raised in goat and conjugated to fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Oakville, ON) was used as the secondary antibody at a dilution of 1:200. For Western blotting, secondary antibody was a horseradish peroxidase conjugated anti-guinea pig IgG employed at 1:100 dilution (Sigma-Aldrich, Oakville, ON).

2.3 - SDS-PAGE and Western blot

SDS-PAGE and Western blotting were performed using standard protocols (Vafopoulou and Steel, 2012). Tissues for SDS-PAGE were dissected and homogenized in lysis buffer and further processed as described in Vafopoulou and Steel (2002). Wells were loaded with homogenate of 10 *Rhodnius* FB lobes; homogenate of 5 *Drosophila* heads were loaded as control. Proteins were separated by 10% acrylamide SDS gel electrophoresis in non-reducing conditions and proteins from unstained gels were transferred electrophoretically onto nitrocellulose filters. Filters were probed with theanti-

PER and the presence of immunoreactive peptide revealed by HRP secondary antibody, using 3,3-diaminobenzidine as the ligand and hydrogen peroxide.

2.4 - Immunohistochemistry (IHC)

The presence of PER protein in fat body cells was investigated using standard immunohistochemical methods, as previously described (Vafopoulou et al., 2010). FB tissue was dissected under *Rhodnius* saline (Lane et al., 1975) and was immediately fixed for 2h in freshly prepared 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.2), washed thoroughly in PBS and incubated in blocking solution for 1 h, all at room temperature. FB was then incubated with the PER antibody overnight at 4 ^{o}C. The tissue was next washed with PBS prior to 2h incubation with secondary antibody. Finally, FB tissue was mounted in glycerol containing 1% 1,4-diazabicyclo[2.2.2] octane (Sigma-Aldrich).

No staining and no autofluorescence were observed when controls were performed in which either the primary or secondary antibody was replaced with non-immune buffer solution.

2.5 - Image collection and analysis

Digital images of 1.0 µm optical sections of FB tissue were taken using an Olympus FV 300 laser scanning confocal microscope. All settings were kept constant. The images were processed using ImageJ 1.41 (NIH), Confocal Assistant 4.02, and Adobe Photoshop CS5. Representative images for each time point were selected and shown. Mean pixel intensity of nuclear and cytoplasmic PER fluorescence was determined using ImageJ ‘line tool.’ Sections of the same length were recorded (3 μm), avoiding the nucleolus and vacuoles, and 3 nuclear as well as 3 cytoplasmic measurements were made per cell, for 10 different cells, from each of the 5 animals per time point (N = 150 for both nucleus and cytoplasm). Cells for measurements were randomly selected by superimposing a transparent paper with markers on the computer screen prior to the image being opened in ImageJ 1.41. The markers served for selecting cells for measurement eliminating any bias. The means ± SEM relative fluorescent intensity measurements were calculated and means were compared using Kruskal-Wallis non-parametric analysis of variance.

2.6 - *In vitro* incubation of fat body

FB from LD and LL larvae were collected under *Rhodnius* saline (Lane et al., 1975) on day 12 after a blood meal. Tissue was washed thoroughly in saline and then incubated *in vitro* in 80 µl antibiotic enriched saline (Vafopoulou and Steel, 1998) containing day 2 *Rhodnius* haemolymph (1:4 ratio of haemolymph to enriched saline) at 28 ± 0.5^{o}C. Different FB were incubated *in vitro* with the enriched saline containing *Rhodnius* day 2haemolymph for 1h, 7h, 13h, 19h, 25h, 31h, 37h or 43h. Incubation medium was removed and replaced with fresh medium every hour. Immediately following *in vitro* incubation tissues were fixed for IHC. In order to assess tissue viability the trypan blue exclusion assay was employed (described in Izumi et al., 2005). Trypan blue dye was dissolved in saline to a final concentration of 0.1%. Tissues were incubated for 2 min at room temperature. Tissue was then rinsed with saline to remove excess dye. Tissue viability was estimated immediately from by blue staining in the tissues (blue stained cell = dead cell). Stained fat body was digitally photographed under a light microscope and percentage of cells stained with trypan blue was calculated using Image J (NIH, Bethesda, MA).

To investigate hormonal regulation of PER expression in FB some samples were provided with a 1 h pulse of either 20-hydroxyecdysone (20E; 3500 ng/ml) or brain media during the 7^{th} h of *in vitro* incubation. Following the 1h hormone pulse FB was washed thoroughly with *Rhodnius* saline and incubations continued as above in antibiotic enriched saline containing day 2 *Rhodnius* haemolymph.

20E (Sigma-Aldrich, Oakville, ON) was dissolved in 100% ethanol and serial dilutions performed in *Rhodnius* saline to the desired physiologically relevant concentration of 3500 ng/mL. This is roughly the maximum hemolymph ecdysteroid titre of a fifth instar larvae on day 12 after a blood meal (Steel et al. 1982). The final concentration of ethanol was <1%.

Brain media were obtained by incubating brain complexes from scotophase day 12 fifth instar larvae in saline for 4h (as described in Vafopoulou and Steel 1996a). The media were partially purified into two fractions (>10 kDa and <10 kDa) by ultrafiltration using Amicon Ultra-0.5 ml (Ultracel-PL 10 kDa membrane) centrifugal tubes (Millipore, Billerica, MA, USA) from 15 brains. These two fractions were re-constituted in *Rhodnius* saline to produce final concentrations of 1 brain equivalent per 80 µl.

3. RESULTS

3.1 - SDS-PAGE and Western blots with anti-PER

Western blotting with the PER antibody revealed the presence of an immunoreactive band of approximately 115 kDa (Fig. 1), the expected molecular weight of PER (Vafopoulou et al. 2010). Another band around 65 kDa was also immunoreactive, which is thought to correspond to a breakdown product of the PER peptide (Vafopoulou et al. 2010). Staining was obviously more intense during the mid-scotophase (Fig. 1, lane A) and weaker during the mid-photophase (Fig. 1, lane B), suggesting daily cycling of the expression of PER in FB. These bands co-migrated with bands in the *Drosophila* control (Fig. 1, lane C). Additionally, identical bands were obtained from larval *Rhodnius* prothoracic gland extract (not shown); these glands have been shown to express PER (Steel and Vafopoulou, 2006). The pattern of staining in all cases corresponds to previous findings using tissue extracts from *Rhodnius* and *Drosophila* employing three different antibodies (Vafopoulou et al., 2010). Overall, these findings indicate that the PER antibody recognizes the native *Rhodnius* PER peptideand suggest that PER may be rhythmically expressed within the FB of *Rhodnius* (see Discussion).

3.2 - *In vivo* localization and cycling of PER in FB

Nuclear and cytoplasmic staining were observed at all time points examined (Fig. 2). Fluorescence intensity in both the cytoplasm and nucleus varied during a day with peak intensity during the scotophase (Fig. 2A, B, E, F) and less intense staining during the photophase (Fig. 2C, D, G, H). This daily rhythm of PER expression was confirmed by quantification of fluorescence intensity (see Materials and Methods) (Fig.2 I) and clearly demonstrated significantly higher fluorescence during the scotophase in both the nucleus (p<0.05) and cytoplasm (p<0.05). In every case, PER immunofluorsecence was consistently higher in the nucleus than the cytoplasm (p<0.05). These results demonstrate that PER cycles with a daily rhythm in *Rhodnius* FB, in both the nucleus and cytoplasm. The phases of cycling of nuclear and cytoplasmic PER-immunoreactivity (PER-IR) were closely similar (see Section 4).

When insects were transferred to continuous darkness (DD), the rhythm of PER immunofluorescence described above for LD animals persisted for three consecutive days with a period length of about 24 hours (Fig. 3). Nuclear and cytoplasmic PER immunofluorescence was observed during all time-points with peak intensity during the scotophase (Fig. 3A, B, E, F) and a diminished intensity during the photophase (Fig. 3C, D, G, H). Quantification of fluorescence intensity (Fig. 3I) revealed similar values to those obtained in LD animals and confirmed a clear rhythm of PER immunofluorescence in both the nucleus and cytoplasm that persisted for three days in DD with damping being observed during the late third day (fig. 3, H, I). Scotophase values were significantly higher than photophase values in both nucleus and cytoplasm (p<0.05). Therefore, the daily rhythm of PER expression observed in LD entrained insects is under circadian control in intact animals.

In order to assess the ability of photic cues to affect PER cycling in FB, insects were maintained in continuous LL for 4 weeks prior to feeding to render them arrhythmic (see Materials and Methods). Only minimal levels of PER immunofluorescence were observed in both the nucleus and cytoplasm of FB from these arrhythmic insects (Fig. 4).

No changes in intensity were detected throughout a 24h period (Fig. 4A-D). These results were confirmed by quantification of fluorescence intensity (Fig. 4M). Transfer of arrhythmic LL animals into DD rapidly induced expression of PER and restored the rhythms of nuclear and cytoplasmic PER immunofluorescence (Fig. 4M). Peak intensities in both cellular compartments were observed during the subjective scotophase (Fig. 4E, F, I) with diminished intensity during the subjective photophase (Fig. 4G, H, K, L) and late subjective scotophase during the second cycle (Fig.4 J). Quantification of intensity levels produced values similar to those obtained for LD animals and confirmed that scotophase values were significantly higher than photophase values in both nucleus and cytoplasm (p<0.05). Transfer of arrhythmic animals to DD was found to induce PER expression that was able to free-run for two cycles, clearly demonstrating that light input is involved, either directly or indirectly, in the regulation of PER in this tissue.

3.3 – Behaviour of PER in FB *in vitro*

It was next investigated whether the circadian cycling of PER originates within FB itself. To do this, FB was dissected and incubated *in vitro* (see section 2). Viability of FB *in vitro* was assessed by the trypan blue exclusion assay. Fat body *in vitro* successfully excluded the dye for up to 32 h. Immediately after dissection and after 8 h of incubation, ~5% of fat body cells were found to have taken up the dye. ~12% of FB cells took up the dye after 14 h of incubation, and ~20% of cells were found dead or dying after 20 and 26 h of *in vitro* incubation. ~30% of FB cells were found to have taken up the trypan blue dye at 32 h of incubation. Lastly, >60% of cells were found to be dead or dying after 38 h of *in vitro* incubation, indicating the tissue was no longer viable. In both L/D entrained and arrhythmic(LL) animals, nuclear and cytoplasmic PER immunofluorescence rapidly declined *in vitro* (Fig. 5 L/D; LL not shown). As early as 1 and 7 hours post-dissection, levels of nuclear PER fluorescence intensity decreased, while cytoplasmic levels were maintained to pre-dissection levels (Fig. 5A, B, I). 13h post *in-vitro* incubation, both nuclear and cytoplasmic PER-IR disappears completely and remain so for the remainder of the incubation, up to 43h (Fig. 5C, D, E, F, G, H). Therefore, *in vitro* incubation of FB led to the loss of PER rhythmicity and, in fact, to the complete cessation of PER expression. These results argue against the presence of an autonomous clock within FB cells (see Discussion).

3.4 – Humoral induction of PER expression in FB *in vitro*

In *Rhodnius*,clocks in the brain and PGs are known to regulate levels of both cerebral neuropeptides (Vafopoulou and Steel, 1996b; Steel and Vafopoulou, 2006) and ecdysteroids (Vafopoulou and Steel, 1998). The possibility that either rhythmic peptide release by the brain or ecdysteroid release by the PGs drives rhythmic PER expression in FB was explored *in vitro*. FB was collected from LL entrained animals and incubated *in vitro* as above. After 7h of incubation, when nuclear PER immunofluorescence decreased, FB was provided with a 1h pulse of a physiologically relevant concentrationof 20HE (see Section 2). Immediately following treatment with 20HE, PER immunofluorescence reappeared in the nucleus and prolonged presence of cytoplasmic PER (Fig. 6). Quantification of PER intensity demonstrated that, as before, post-20HE administration, nuclear PER intensity was significantly higher than cytoplasmic PER intensity (p<0.05) (Fig. 6I). Additionally, maximum intensity values reached following the 1h pulse of 20HE were lower than those observed during the scotophase *in vivo* (see and compare to Fig. 2). The levels of PER fluorescence obtained after 20HE treatment were maintained for 6 hours (Fig. 6D), but nuclear intensity significantly declined by 12h following the 20HE pulse (Fig. 6E) and subsequent time points showed no detectable PER fluorescence in either nucleus or cytoplasm (Fig. 6, F, G, H). These results argue that PER expression is induced by 20HE in FB.

Similar *in vitro* incubations of FB were performed during which, after 7h *in vitro*, a 1h pulse of either <10 kDa or >10 kDa brain media fractions (see Section 2) was provided. Neither the <10 kDa fraction (Fig. 7) nor the >10 kDa fraction (Fig. 8) had any effect on PER expression by FB *in vitro*. In both cases, nuclear PER immunofluorescence rapidly declined over the first 7h *in vitro,* after which both nuclear and cytoplasmic PER presence decreased and became undetectable for the rest of the 38h incubation period. These results are identical to those obtained by *in vitro* incubation of untreated FB (Fig. 5), demonstrating that neither fraction has any direct effect on PER expression in this tissue. Potential synergistic effects of 20HE and brain media fractions were assessed by repeating the *in vitro* incubation procedure with a 1h pulse of either both 20HE and <10 kDa brain media fraction, 20HE and >10 kDa brain media fraction or whole brain media. The first two treatments mimicked the effects of the 1h pulse of 20HE alone (Fig. 9, S, T). Incubation with 20-Hydroxyecdysone (3500 ng/mL) and <10kDa brain neuropeptide fraction (equivalent to 1 *Rhodnius* brain) for 1h was found to induce nuclear and cytoplasmic PER expression 1h and 7h post-incubation (Fig. 9 A and D, respectively). Similar results were achieved when incubating with 20-Hydroxyecdysone (3500 ng/mL) and >10kDa brain neuropeptide fraction (equivalent to 1 *Rhodnius* brain) for 1h (Fig. 9 B, E). In both cases, 6h later, nuclear PER levels were found to be abolished (G, H). 6h afterwards, cytoplasmic levels disappeared as well (J, K), and remained as such for the next 12h (M, P, N, Q). However, incubation with 1 whole *Rhodnius* brain equivalent (>10kDa + <10kDa) for 1h, was found to have no effect on PER expression at any time point for the next 31h (C, F, I, L, O, R). No synergistic effects were observed.

Figure 1 Western blot of L5 *Rhodnius* fat body and *Drosophila* head extract**.** L5 *Rhodnius* FB extract wasfrom day 12 post-blood meal. Proteins were separated in 10% SDS-PAGE. Arrow on left marks the position of the 115 kDa peptide immunoreactive to Genscript PER antibody staining of scotophase and photophase fat body (A and B, respectively) and *Drosophila* head extract (C). 10 fat body lobes and 5 *Drosophila* heads were used per well.

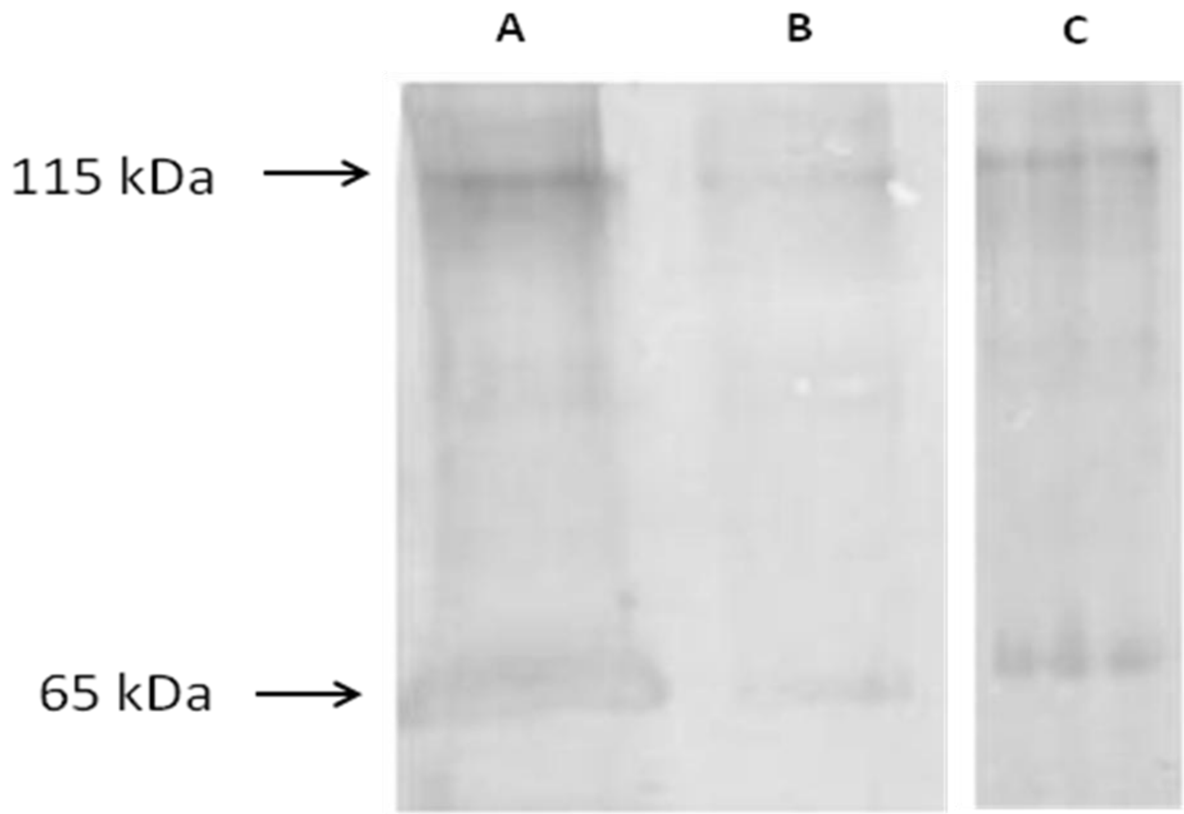

A
B
C
115 kDa
65 kDa

Figure 2 Daily cycling of PER in the nucleus and cytoplasm of fat body cells**.** Daily changes in the intensity of PER immunofluorescence in the fat body of the fifth instar larva in LD at 12 and 13 days after a blood meal. Arrow points to nuclear PER. Scale bar = 10 μm. Dark bars indicate darkness, light bars indicate light. The dashed vertical line separates days. Points are mean +/- SEM (N=150).

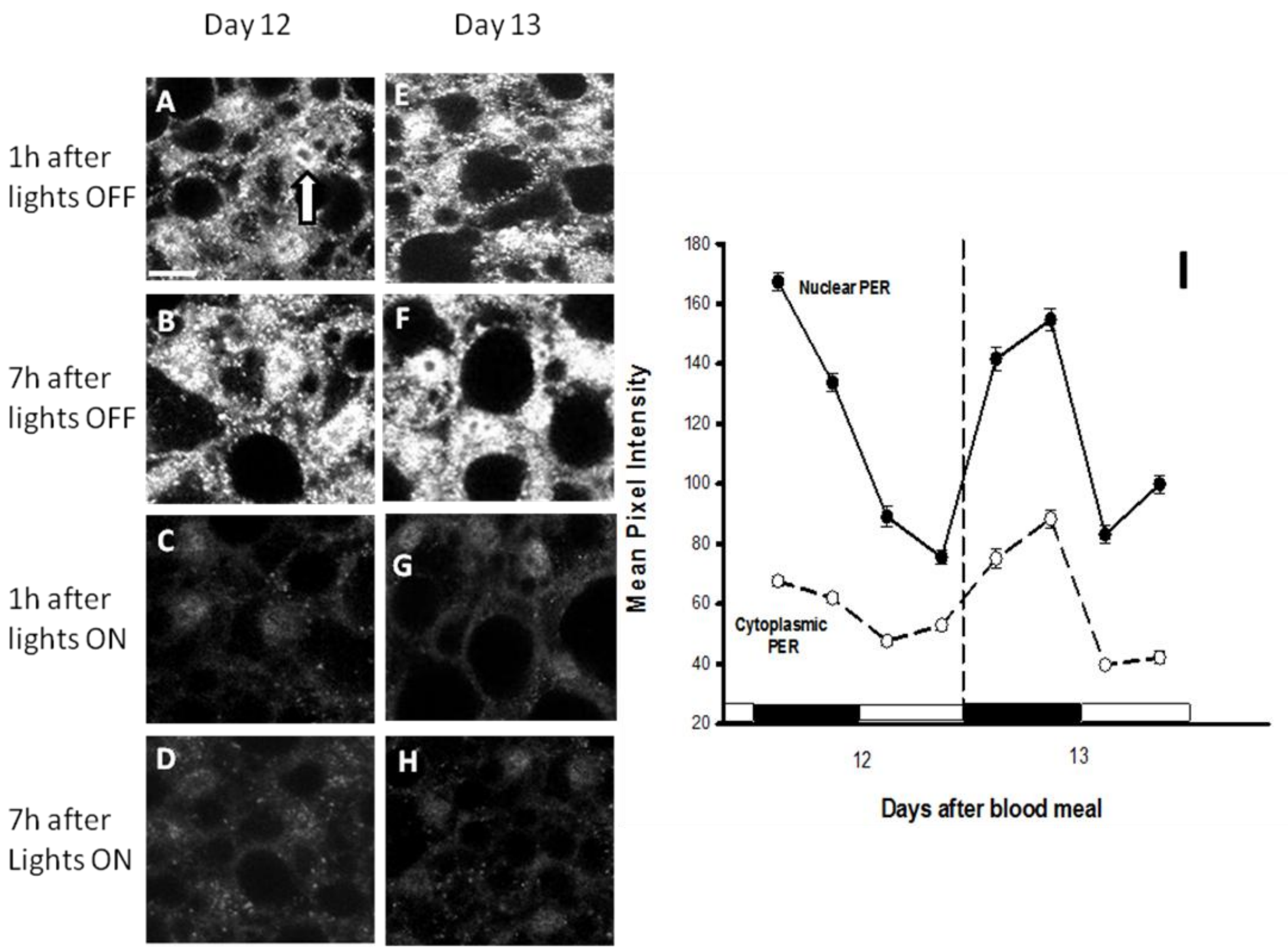
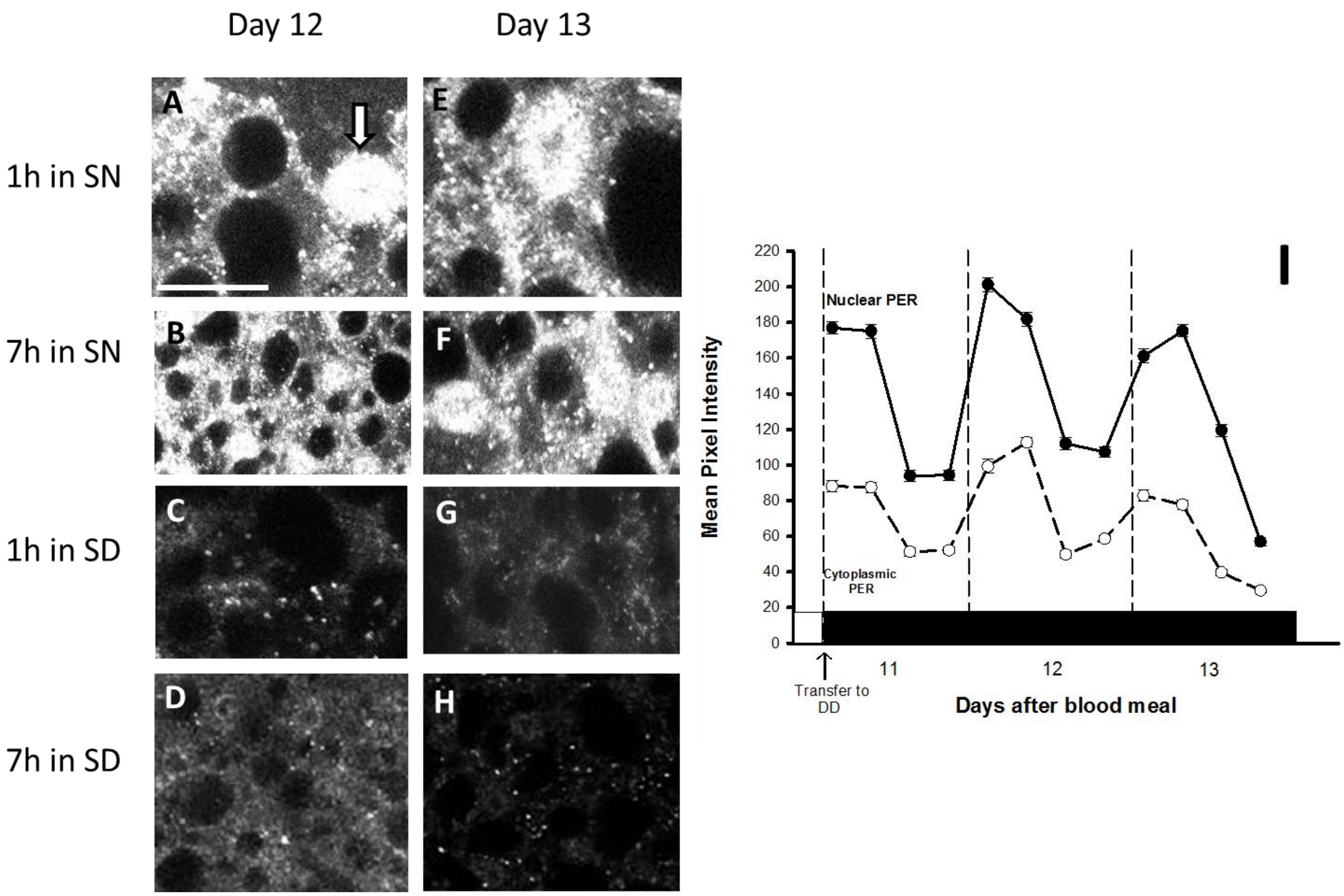

Day 12
Day 13
A
B
C
D
E
F
G
H
1h after lights OFF
7h after lights OFF
1h after lights ON
7h after Lights ON
Nuclear PER
Cytoplasmic PER
Mean Pixel Intensity
180
160
140
120
100
80
60
40
20
12
13
Days after blood meal

Figure 3 Daily cycling of PER persists in aperiodic conditions (DD)**.** Daily changes in the intensity of PER immunofluorescence in the fat body of the fifth instar larva at 12 and 13 days after a blood meal.LD Animals were transferred to a DD regime at day 10 post-blood meal. Arrow points at nuclear PER. Scale bar = 10 μm. Dark bars indicate darkness, and the dashed vertical lines separate days.Points are mean +/- SEM (N=150).



Day 12
Day 13
1h in SN
7h in SN
1h in SD
7h in SD
A
B
C
D
E
F
G
H
I
Nuclear PER
Cytoplasmic PER
Mean Pixel Intensity
220
200
180
160
140
120
100
80
60
40
20
0
11
12
13
Transfer to DD
Days after blood meal

Figure 4 Cycling of non-rhythmic PER in arrythmic animals restored by transfer to darkness**.** Daily changes in the intensity of PER immunofluorescence in the fat body of the fifth instar larva at 12 and 13 days after a blood meal. Arrhythmic animals exposed to a constant light (LL) regime for >4 weeks were transferred to continuous darkness (DD). Arrow points at nuclear PER. Scale bar = 10 μm. Dark bars indicate darkness, light bars indicate light. The dashed vertical line separates days. Points are mean +/- SEM (N=150).

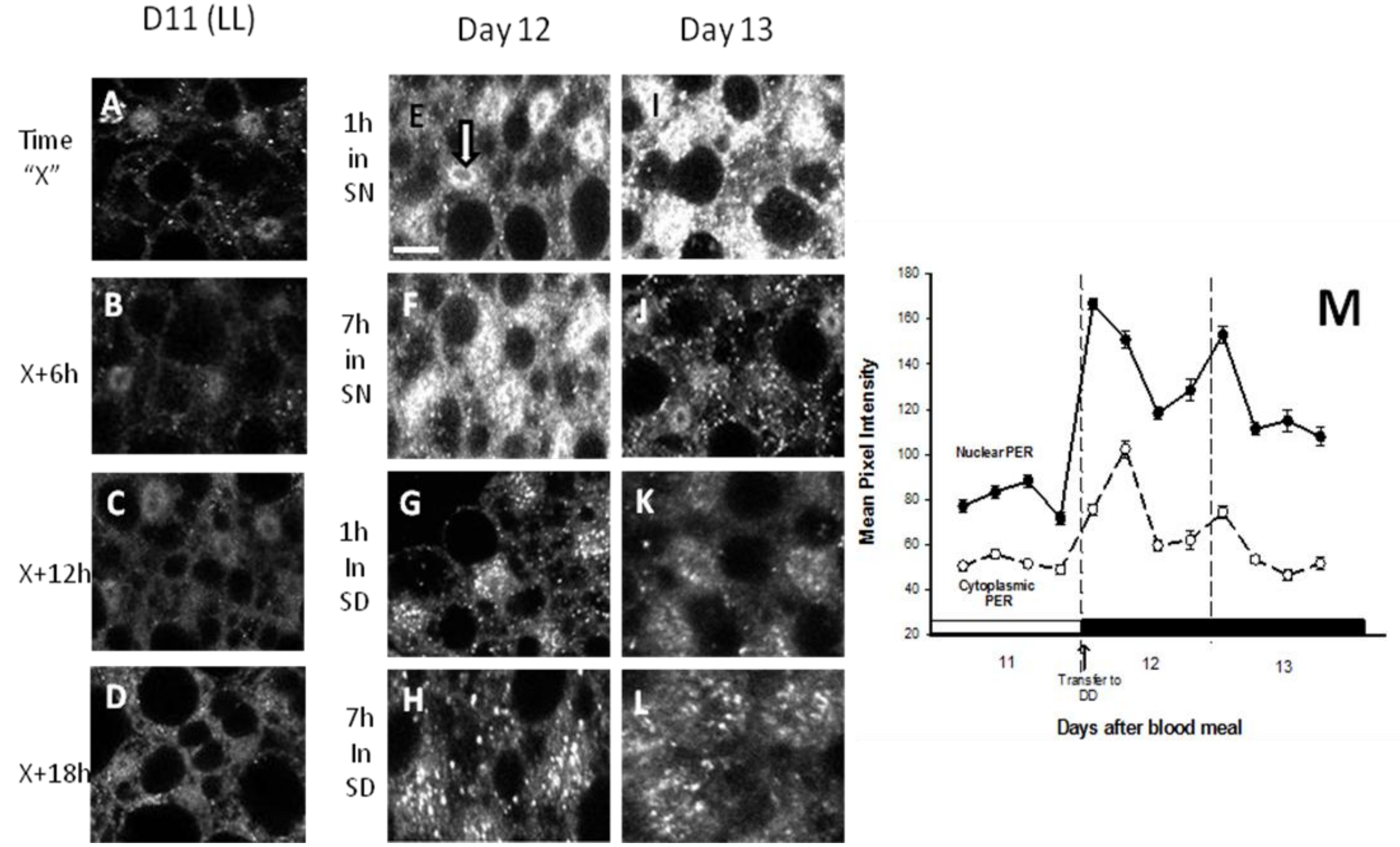

D11 (LL)
Day 12
Day 13
Time "X"
X+6h
X+12h
X+18h
1h in SN
7h in SN
1h In SD
7h In SD
A
B
C
D
E
F
G
H
I
J
K
L
M
Mean Pixel Intensity
Nuclear PER
Cytoplasmic PER
Transfer to DD
Days after blood meal
11
12
13
180
160
140
120
100
80
60
40
20

Figure 5 PER expression and rhythm are lost in FB *in vitro***.** Intensity of PER immunofluorescence in the fat body of the fifth instar larva. Fat body lobes were dissected at day 12 from insects in an LD regime and incubated *in vitro* in LD with *Rhodnius* ringers with antibiotic. Arrow points at nuclear PER. Scale bar = 10 μm. Dark bars indicate darkness, light bars indicate light. The dashed vertical line separates days. Faint staining of PER was observed at times for which there is only one data point instead of two for nuclear and cytoplasmic PER. No difference between nuclear and cytoplasmic PER was measured at these time points. Points are mean +/- SEM (N=150).

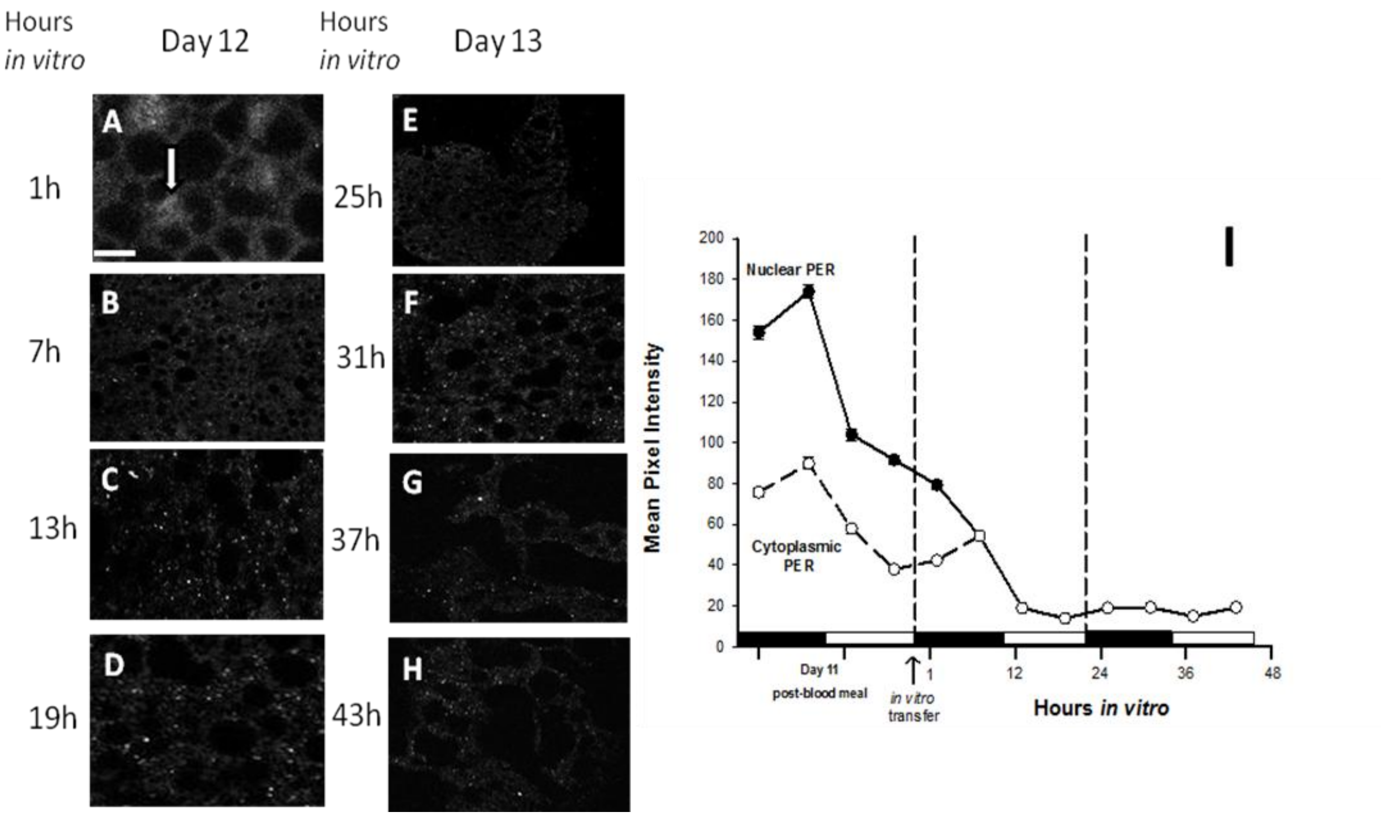

Hours
in vitro
Day 12
Hours
in vitro
Day 13
A
B
C
D
E
F
G
H
1h
7h
13h
19h
25h
31h
37h
43h
I
Nuclear PER
Cytoplasmic
PER
Mean Pixel Intensity
Day 11
post-blood meal
in vitro
transfer
Hours in vitro

Figure 6 A 1 h pulse of ecdysteroid induces FB PER expression *in vitro***.** Intensity of PER immunofluorescence in the fat body of arrhythmic (LL) fifth instar larva at 12 and 13 days after a blood meal was measured under LL conditions *in vitro*. Arrow points at nuclear PER. Scale bar = 10 μm. Light bar indicates light. The dashed vertical line represents time of pulse administration. Faint staining of PER was observed at times for which there is only one data point instead of two for nuclear and cytoplasmic PER. No difference between nuclear and cytoplasmic PER was measured at these time points. Points are mean +/- SEM (N=150).

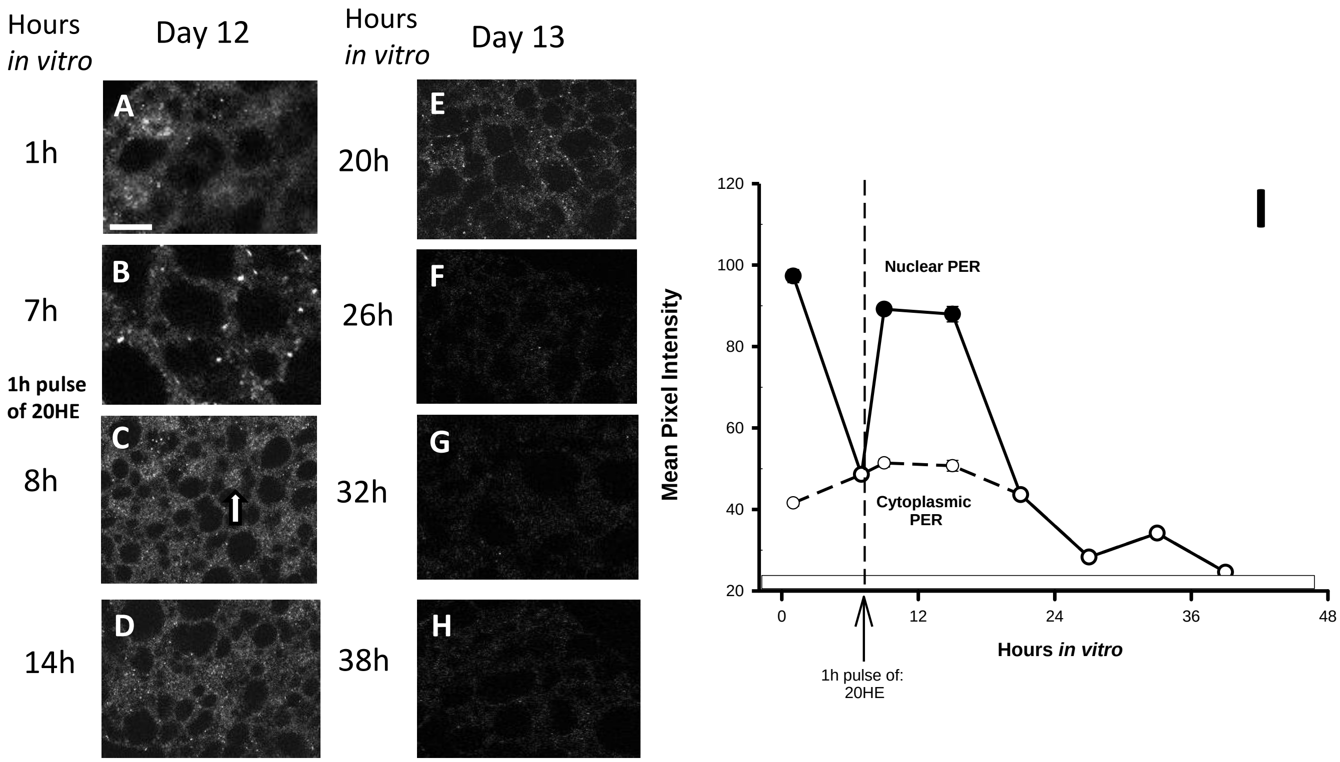
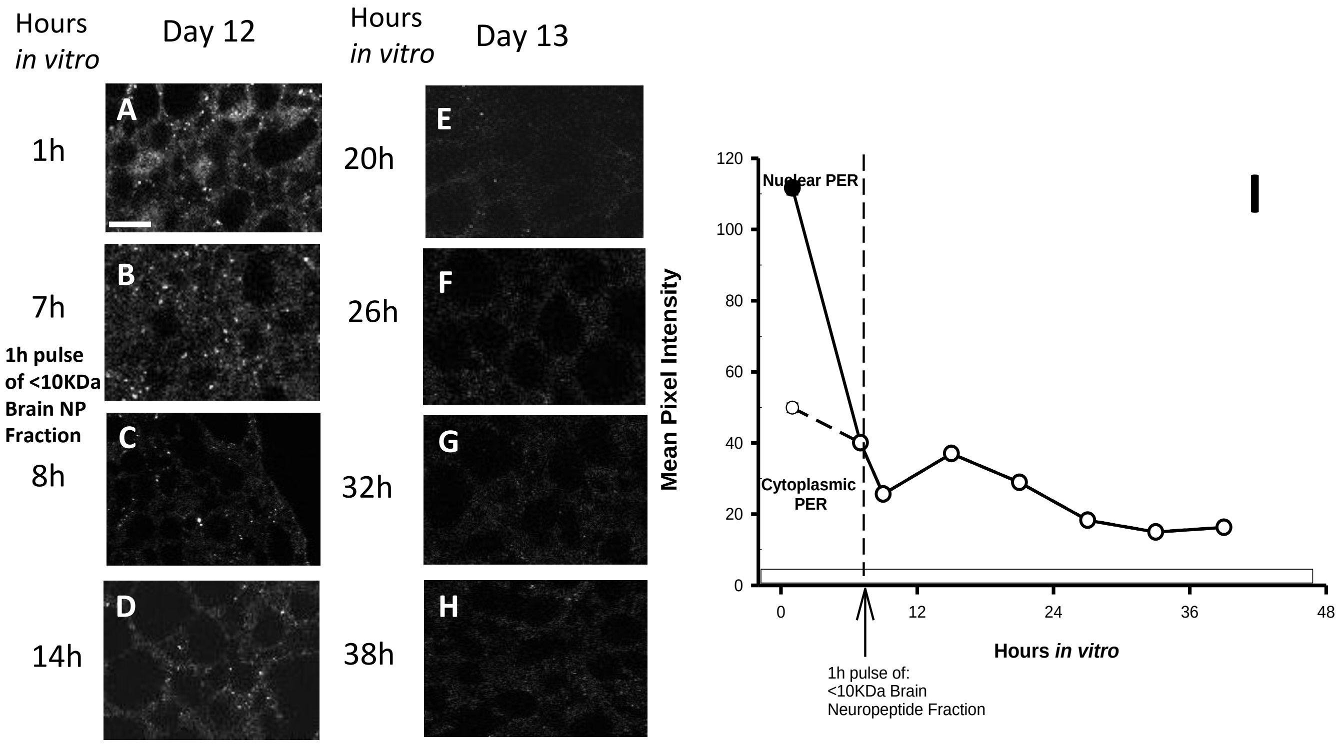
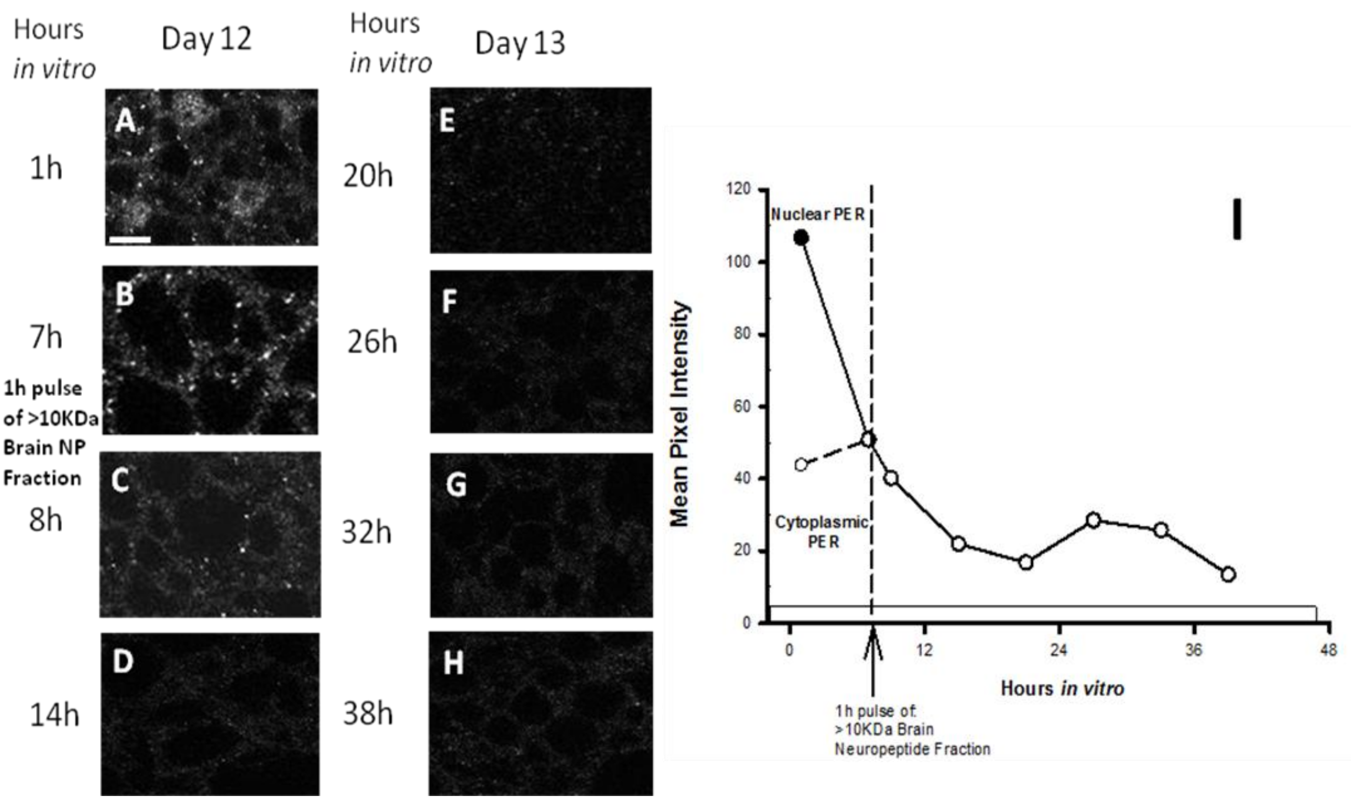

Hours
in vitro
Day 12
Hours
in vitro
Day 13
1h
A
20h
E
7h
B
26h
F
1h pulse
of 20HE
8h
C
32h
G
14h
D
38h
H
I
Nuclear PER
Cytoplasmic
PER
Mean Pixel Intensity
120
100
80
60
40
20
0
12
24
36
48
Hours in vitro
1h pulse of:
20HE

Figure 7 A 1 h pulse of <10kDa brain neuropeptide fraction had no effect with respect to PER expression by the FB *in vitro*. Intensity of PER immunofluorescence in the fat body of arrhythmic (LL) fifth instar larva at 12 and 13 days after a blood meal was measured under LL conditions *in vitro*. Scale bar = 10 µm. The dashed vertical line represents time of pulse administration. Faint staining of PER was observed at times for which there is only one data point instead of two for nuclear and cytoplasmic PER. No difference between nuclear and cytoplasmic PER was measured at these time points. Points are mean +/- SEM (N=150).



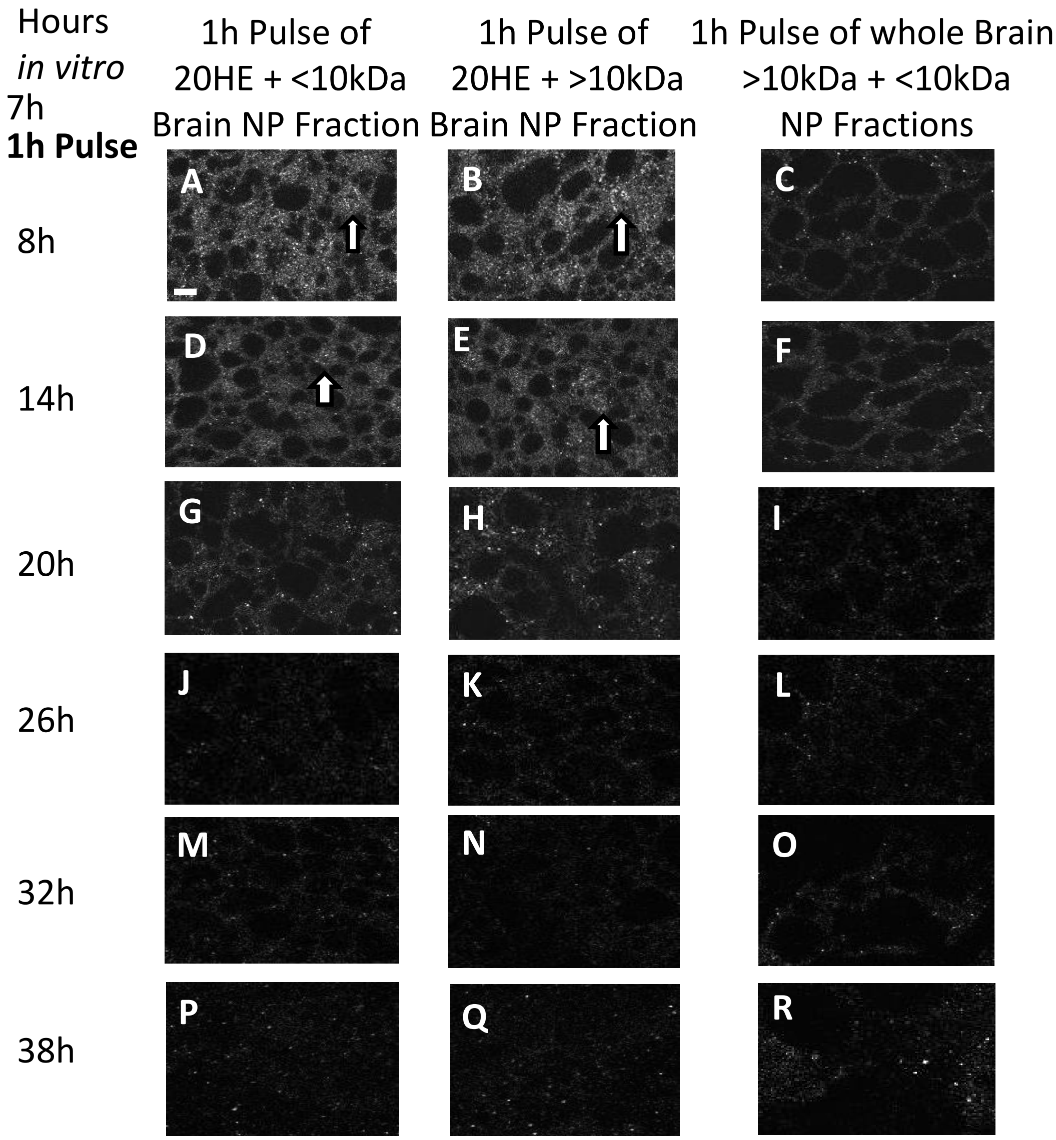
Hours
in vitro
Day 12
Hours
in vitro
Day 13
1h
A
20h
E
7h
B
26h
F
1h pulse
of <10KDa
Brain NP
Fraction
8h
C
32h
G
14h
D
38h
H
Mean Pixel Intensity
Nuclear PER
Cytoplasmic
PER
Hours in vitro
1h pulse of:
<10KDa Brain
Neuropeptide Fraction

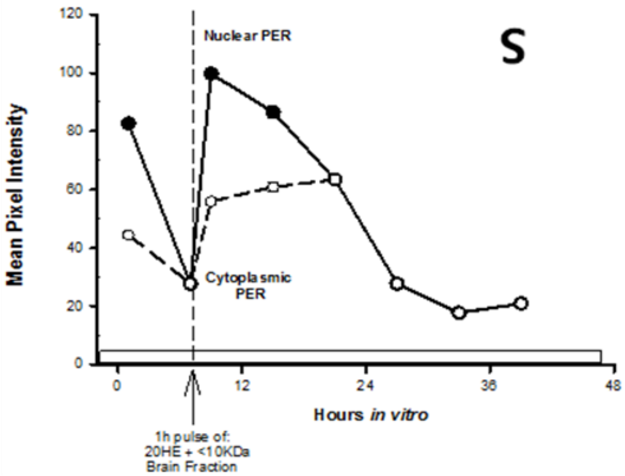
Figure 8 A 1 h pulse of >10kDa brain neuropeptide fraction had no effect with respect to PER expression by the FB *in vitro***.** Intensity of nuclear PER immunofluorescence in the fat body of arrhythmic (LL) fifth instar larva at 12 and 13 days after a blood meal was measured under LL conditions *in vitro*. Scale bar = 10 μm. The dashed vertical line represents time of pulse administration. Faint staining of PER was observed at times for which there is only one data point instead of two for nuclear and cytoplasmic PER. No difference between nuclear and cytoplasmic PER was measured at these time points. Points are mean +/- SEM (N=150).

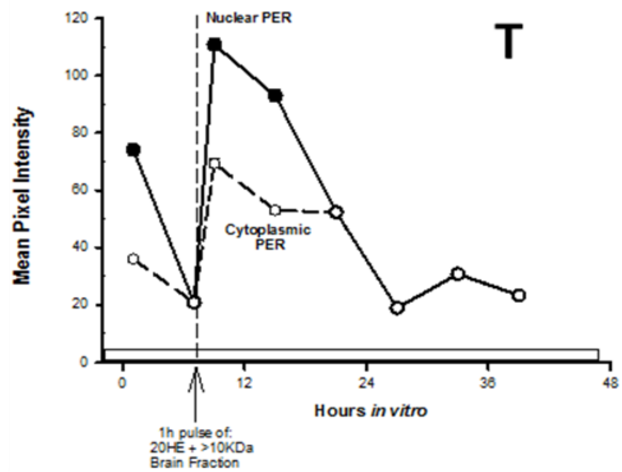


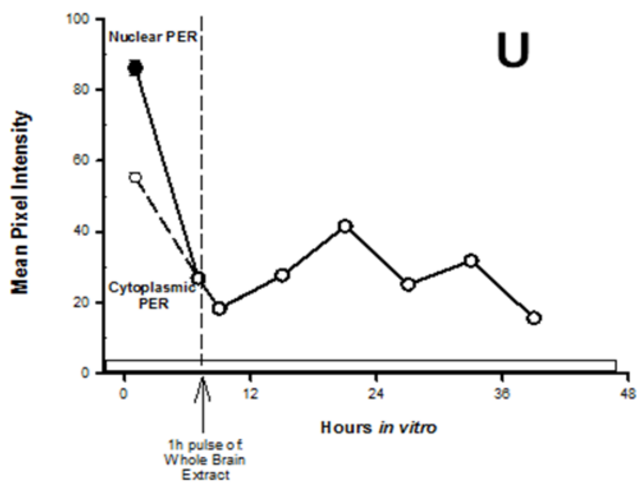
Hours
in vitro
Day 12
Hours
in vitro
Day 13
A
1h
20h
E
B
7h
26h
F
1h pulse
of >10KDa
Brain NP
Fraction
C
8h
32h
G
D
14h
38h
H
Nuclear PER
Cytoplasmic
PER
I
Mean Pixel Intensity
Hours in vitro
1h pulse of:
>10KDa Brain
Neuropeptide Fraction

Figure 9 No synergistic effects were observed between ecdysteroid, <10kDa and >10kDa brain neuropeptide fractions with respect to PER expression by the FB *in vitro***.** Intensity of PER immunofluorescence in the fat body of arrhythmic (LL) fifth instar larva at 12 and 13 days after a blood meal was measured under LL conditions *in vitro*. Arrows point at nuclear PER. Scale bar = 10 μm. The dashed vertical line represents time of pulse administration. Faint staining of PER was observed at times for which there is only one data point instead of two for nuclear and cytoplasmic PER. No difference between nuclear and cytoplasmic PER was measured at these time points. Points are mean +/- SEM (N=150).


Hours
in vitro
7h
1h Pulse
1h Pulse of 20HE + <10kDa Brain NP Fraction
1h Pulse of 20HE + >10kDa Brain NP Fraction
1h Pulse of whole Brain >10kDa + <10kDa NP Fractions
8h
14h
20h
26h
32h
38h
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R


S
Nuclear PER
Cytoplasmic PER
Mean Pixel Intensity
Hours *in vitro*
1h pulse of:
20HE + <10KDa
Brain Fraction


T
Nuclear PER
Cytoplasmic PER
Mean Pixel Intensity
Hours *in vitro*
1h pulse of:
20HE + >10KDa
Brain Fraction


U
Nuclear PER
Cytoplasmic PER
Mean Pixel Intensity
Hours *in vitro*
1h pulse of:
Whole Brain
Extract

4. DISCUSSION

FB of insects is analogous to the mammalian liver and adipose tissue, serving a wide range of functions ranging from metabolism to energy storage and detoxification (Reviewed in Arrese and Soulages, 2010). Circadian coordination of these functions in the FB is crucial in order for the animal to achieve homeostatic balance at all times during the daily cycle. We investigated whether *Rhodnius* FB possesses a circadian clock by observing expression of PER protein throughout the 24 hour day. SDS-PAGE and Western blot analysis, using a custom made PER antibody revealed the presence of an 115kDa peptide in fat body that was confirmed to be native *Rhodnius* PER. When this antibody was employed with immunohistochemistry and laser confocal microscopy, PER-IR was observed to cycle in fat body cells *in vivo*. PER-IR was seen in both cytoplasm and nucleus, at all time points, with peak intensity during the scotophase, higher than photophase values. These results are consistent with the current model of the molecular oscillator. When insects were transferred to constant darkness PER cycling continued for at least 3 cycles with a period of roughly 24 hours, and damping of PER was noted in the third cycle. Additionally, arrhythmic animals generated by prolonged exposure to constant light conditions, had only low and constant levels of PER, ie. the *in vivo* PER rhythm in the fat body was lost. However, transfer of these arrhythmic animals to DD, restored the rhythm of PER cycling, showing that PER within the fat body can be induced *in vivo* by light, directly or indirectly. Together, these results indicate that PER cycling in fat body *in vivo* is under endogenous circadian control.

Circadian PER expression in fat body *in vivo* may be generated by a local clock in the fat body itself, or driven by clocks located elsewhere in the animal. When fat body was incubated *in vitro*, not only was the PER rhythm lost, but the tissue was found to be entirely depleted of PER within 12 hours. The loss of PER-IR *in vitro* cannot be due to the death of the tissue since the trypan blue exclusion assay showed that tissue remained viable for at least 32 hours of *in vitro* incubation. Therefore, fat body is incapable of self-sustained PER cycling. The rhythmic PER expression and response to photic cues observed *in vivo* must be regulated by a clock located outside of the fat body.

In insects, fat body is not innervated and therefore cycling PER expression in this tissue must be under endocrine control. In *Rhodnius,* several hormones have been demonstrated to be regulated by the circadian system. A circadian rhythm of PTTH release from the brain occurs throughout fifth instar larval development. ILPs were shown to be synthesized and released by cerebral neurosecretory cells in a circadian fashion (Vafopoulou and Steel, 2012). Both PTTH and ILPs are under direct control of the *Rhodnius* brain clock. Further, a circadian rhythm of ecdysteroids in the haemolymph is generated by an autonomous circadian clock in the prothoracic glands (Ampleford and Steel, 1986; Vafopoulou and Steel, 1998). Therefore, the possibility exists that circadian PER expression in fat body *in vivo* is driven by rhythms of neuropeptides and/or steroid hormones.

Fat body from arrhythmic animals *in vitro* was exposed to a brief pulse of a physiologically relevant concentration of 20HE (see Section 2) at the start of incubation. PER expression was induced 1 hour thereafter, and persisted for 6 hours, when it declined over the next 6 hours, until becoming completely undetectable. Therefore, 20HE is capable of inducing PER expression *in vitro*, and since 20HE was removed from the incubating media after 1 hour, 20HE presence appears to be required for PER expression by the fat body. While this is the first report of 20HE-induced PER expression, 20HE has been previously shown to have a role in regulating *vrille*, a clock gene, expression in *Drosophila*. Itoh et. al., 2011 found that *vrille* expression is, at least in part, controlled by circulating ecdysteroids through EcR.

In *Rhodnius*, it is known that the circadian rhythm of circulating ecdysteroids peaks during the scotophase (Ampleford and Steel, 1986), corresponding to the observed peak of PER expression in fat body *in vivo.* Also, the observed damping of PER in the third cycle post transfer to constant darkness coincides and could be explained by the known damping of the ecdysteroids rhythm in such conditions.Further, the ecdysteroid receptor EcR is present in fat body and exhibits a circadian rhythm within this tissue that also peaks during the scotophase (Vafopoulou and Steel, 2006). Therefore, it is very probable that the rhythm of PER expression in fat body *in vivo* is induced and driven by this known circadian rhythm of ecdysteroids in the haemolymph.

PER-IR induced by 20HE in fat body *in vitro* was less intense than that observed *in vivo*, suggesting that other factors may be involved in the regulation of PER expression *in vivo.* In order to address this observation, as well as determine whether the brain clock has a direct pathway of temporal control to this tissue, *in vitro* fat body was exposed to brain neuropeptide extract for a brief 1 hour pulse 7 h after incubation, at which point PER-IR was no longer detectable. Brain neuropeptide extract was separated into >10 kDa and <10 kDa fractions; PTTH and bombyxin would be included in the >10 kDa and <10 kDa, respectively (Vafopoulou and Steel, 2002). Both PTTH and bombyxin have been shown to modulate the PG peripheral clock (Vafopoulou and Steel, 1999 and unpublished results from our lab) and therefore it is not unreasonable to expect they might affect PER expression in the fat body. However, neither whole brain neuropeptide extract nor the individual fractions induced PER expression in the fat body *in vitro*, showing that the output of the brain clock does not act directly on PER expression in the fat body. Addition of 20HE to the individual fractions led to the successful induction of PER expression *in vitro.*Therefore, the presence of ecdysteroid appears to be essential in order to induce PER expression in the fat body. It is inferred that the output of the brain clock does not play a direct role in the induction of PER expression in the fat body, but rather, that it conveys temporal information to the fat body through its modulatory effect of the PG clock and ecdysteroid secretion.

A potential mechanism of circadian control of PER expression in fat body *in vivo* emerges from these findings. It is suggested that the brain clock indirectly provides temporal information to the fat body, driving PER expression in a circadian fashion. The brain clock is known to release PTTH with a circadian rhythm, which in turn controls the phase of the PG peripheral clock and its synthesis of ecdysteroids (Vafopoulou and Steel, 1999). Subsequently, these ecdysteroids are released into the haemolymph during the scotophase. *Rhodnius* fat body is a known target of ecdysteroids and exhibits a circadian rhythm of EcR expression (Vafopoulou and Steel, 2006). Ecdysteroids are therefore able to act on fat body to induce PER expression in a circadian fashion. A strikingly similar analogy with the mammalian HPA-axis becomes immediately apparent. The SCN drives rhythms of tropic peptide hormones, released from the hypothalamus-pituitary system and thus regulates a circadian rhythm of steroid hormone (ie. glucocorticoids) release by the adrenal glands. The liver, functionally analogous to the insect fat body is a known target of rhythmic glucocorticoids. Both glucocorticoids and ecdysteroids subsequently act as messengers of time to target tissues to establish temporal order within the organism. In this way the circadian system can precisely orchestrate the diverse functions of the fat body during the 24 hour day.

The concept of a slave oscillator driven by rhythmic outputs generated by a master pacemaker originally arose as a theoretical construct explaining early results in the field (Pittendrigh, 1974). The current view of circadian organization in animals is that a central clock in the brain regulates/modulates autonomous peripheral clocks throughout the organism. Our results demonstrate that in insects peripheral oscillators can be present with a range of autonomy. In *Rhodnius,* the brain clock remains the master timekeeper and integrator of timing information regulating rhythms in behavioral and hormonal outputs (i.e. PTTH in *Rhodnius*). These hormonal outputs then entrain (set the phase of) autonomous clocks such as the PGs. Indirectly, setting the phase of the PG clock will coordinate the circadian concentration of haemolymph ecdysteroid, which then drives the rhythms of clock gene expression in peripheral tissues such as the fat body. The rhythmic induction of clock genes by hormones may be more common than is currently understood considering the potential lack of autonomy of many assumed peripheral clocks. This range of clock autonomy presumably provides the circadian system more subtle control of temporal organization.

5. Acknowledgements

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REFERENCES

Ampleford E, Steel CGH. 1982. Circadian control of ecdysis in *Rhodnius prolixus* (Hemiptera). *J Comp Physiol* **147:** 281-286.

Ampleford EJ, Steel CGH. 1986. Circadian control of a daily rhythm in hemolymph ecdysteroid titer in the insect *Rhodnius prolixus* (Hemiptera). *Gen Comp Endocrinol* **59(3):** 453-459.

Arrese EL, Soulages JL. 2010. Insect fat body: energy, metabolism and regulation. *Annu Rev Entomol* **55:** 207-225.

Balsalobre A, Damiola F, Schibler U. 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93:** 929–937.

Britton JS, Lockwood WK, Li L, Cohen SM, Edgar BA. 2002. *Drosophila*'s insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. *Dev Cell* **2:** 239–249.

Buijs RM, Kalsbeek A. 2001. Hypothalamic integration of central and peripheral clocks. *NatureReviews Neurosci* 2:521–526.

Colombani J, Bianchini L, Layalle S, Pondeville E, Dauphin-Villemant C, Antoniewski C, Carre C, Noselli S, Leopold P. 2005. Antagonistic actions of ecdysone and insulin determine final size in Drosophila. *Science* 310:667–670.

Glinka AV, Wyatt GR. 1996. Juvenile hormone activation of gene transcription in locust fat body. *Insect Biochem Mol Biol* **26:** 13–18.

Hardin PE. 2009. Molecular mechanisms of circadian timekeeping in *Drosophila*. *Sleep Biol Rhythm* **7(4):** 235-242.

Hastings MH, O'Neill JS, Maywood, ES. 2007. Circadian clocks: regulators of endocrine and metabolic rhythms. *J Endocrinol* **195:** 187-198.

Itoh TQ, Tanimura T, Matsumoto A. 2011. Membrane-bound transporter controls the circadian transcription of clock genes in *Drosophila*. *Genes Cells* **16:** 1159-1167.

Izumi Y, Sonoda S, Yoshida H, Tsumuki H. 2005. Identification of tissues showing the lowest tolerance to freezing in larvae of the rice stem borer, *Chilo suppressalis*. *Physiol Entomol* **30:** 324-331.

Jensen Al, Brasch K. 1985. Nuclear development in locust fat body: the influence of juvenile hormone on inclusion bodies and the nuclear matrix. *Tissue Cell* **17(1):**117-130.

Lane NJ, Leslie RA, Swales LS. 1975. Insect peripheral nerves: accessibility of neurohaemal regions to lanthanum. *J Cell Sci* **18:**179-197.

Liu Y, Liu H, Liu S, Wang S, Jiang RJ, Li S. 2009. Hormonal and nutritional regulation of insect fat body development and function. *Arch Insect Biochem Physiol* **74(1):**16-30.

Pelc D, Steel CGH. 1997. Rhythmic steroidogenesis by the prothoracic glands of the insect *Rhodnius prolixus* in the absence of rhythmic neuropeptide input: implications for the role of prothoracicotropic hormone. *Gen Comp Endocrinol* **108(3):**358-365.

Pittendrigh CS. 1974. Circadian oscillations in cells and the circadian organization of multicellular systems. In: Schmitt FO, Worden GO (eds) *The Neurosciences: Third Study Program*, pp. 437-458. Cambridge: MIT Press.

Rajathi A, Pandiarajan J, Krishnan M. 2010. Effect of RH-2485 on development, metamorphosis and synthesis of major proteins in female silkworm *Bombyx mori*. *Biologia* **65(5):**903-913.

Riddiford LM, Hiruma K, Zhou X, Nelson CA. 2003. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. *Insect Biochem Mol Biol* **33:**1327–1338.

Steel CGH, Vafopoulou X. 2006. Circadian orchestration of developmental hormones in the insect, *Rhodnius prolixus*. *Comp Biochem Physiol A* **144:**351-364.

Steel CGH, Bollenbacher WE, Smith SL, Gilbert LI. 1982. Haemolymph ecdysteroid titres during larval-adult development in *Rhodnius prolixus*: correlations with moulting hormone action and brain neurosecretory cell activity. *J Insect Physiol* **38:**519-525.

Vafopoulou X, Steel CGH. 1991. Circadian regulation of synthesis of ecdysteroids by prothoracic glands of the insect *Rhodnius prolixus*: Evidence of a dual oscillator system. *Gen Comp Endocrinol* **83(1):**27-34.

Vafopoulou X, Steel CGH. 1992. *In vitro* photosensitivity of ecdysteroid synthesis by prothoracic glands of *Rhodnius prolixus* (Hemiptera). *Gen Comp Endocrinol* **86(1):**1-9.

Vafopoulou X, Steel CGH. 1996a. The insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: re-evaluation of its role in development. *Proc Nat Acad Sci USA* **93(8):**3368-3372.

Vafopoulou X, Steel CGH. 1996b. Circadian regulation of a daily rhythm of release of prothoracicotropic hormone from the brain-retrocerebral complex of *Rhodnius prolixus* (Hemiptera) during larval-adult development. *Gen Comp Endocrinol* **102(1):**123-129.

Vafopoulou X, Steel CGH. 1998. A photosensitive circadian oscillator in an insect endocrine gland: photic induction of rhythmic steroidogenesis *in vitro*. *J Comp Physiol A* **182(3):**343-349.

Vafopoulou X, Steel CGH. 1999. Daily rhythm of responsiveness to prothoracicotropic hormone in prothoracic glands of *Rhodnius prolixus*. *Arch Insect Biochem Physiol* **41:**117-123.

Vafopoulou X, Steel CGH. 2002. Prothoracicotropic hormone of *Rhodnius prolixus*: partial characterization and rhythmic release of neuropeptides related to Bombyx PTTH and bombyxin. *Inver Rep Dev* **42(2):**111-120.

Vafopoulou X, Steel CGH. 2006. Ecdysteroid hormone nuclear receptor (EcR) exhibits circadian cycling in certain tissues, but not others, during development in *Rhodnius prolixus* (Hemiptera). *Cell Tissue Res* **323(3):**443-455.

Vafopoulou X, Steel CGH. 2012. Insulin-like and testis ecdysiotropin neuropeptides are regulated by the circadian timing system in the brain during larval-adult development in the insect *Rhodnius prolixus* (Hemiptera). *Gen Comp Endocrinol* **179(2):**277-288.

Vafopoulou X, Terry KL, Steel CGH. 2010. The circadian timing system in the brain of the fifth larval instar of *Rhodnius prolixus* (Hemiptera). *J Comp Neurol* **518:**1264-1282.

Xu K, DiAngelo JR, Hughes ME, Hogenesch JB, Sehgal A. 2011. Interaction between circadian clocks and metabolic physiology: implications for reproductive fitness. *Cell Metab* **13(6):**639-654.

Xu K, Zheng X, Sehgal A. 2008. Regulation of feeding and metabolism by neuronal and peripheral clocks in *Drosophila*. *Cell Metab* **8(4):**289-300.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, Tei H. 2000. Resetting central and peripheral circadian oscillators in transgenic rats. *Science* **288:** 682–685.

Zhao Z, Zera AJ. 2004. The hemolymph JH titer exhibits a large-amplitude, morph-dependent, diurnal cycle in the wing-polymorphic cricket, *Gryllus firmus*. *J Insect Physiol* **50:** 93-102.

CHAPTER III: GENERAL DISCUSSION

3.1 - Rhythmic PER suggestive of a potential peripheral clock

I have shown via both western blotting and IHC that in normal L/D conditions, PER is present in the fat body of *Rhodnius,* and exhibits daily cycling, with a peak in scotophase and trough in photophase. Further, IHC data shows that during the night-time, there is greater cytoplasmic accumulation of PER, as well as more nuclear translocation, both consistent with the known molecular oscillator model (see Introduction). However, cycling PERalone is not necessarily conclusive evidence of an existing autonomous clock in the fat body (see below).

In the early days of circadian biology, the main belief was that slave oscillators exist in different tissues throughout the body and that a master clock in the brain drives and regulates all the other clocks (Pittendrigh, 1974). This view was believed to hold true in a myriad of species, including flies, bugs, and vertebrates (Tomioka and Abdelsalam, 2004). The identification of clock genes such as *per* and *tim*, *clk* and cyc (or *bmal1*) (see Introduction), gave researchers tools with which to identify where potential clocks are located. Expression of clock genes was found to be under circadian control in many peripheral tissues and believed to be under the control of this master clock (Hastings et al., 2007). However, a discovery by Balsalobre and colleagues challenged this view. They found that fibroblasts continue to express clock gene rhythms, in *in vitro* cultures (Balsalobre et al., 1998). Since then, it has been shown that most major organs in vertebrates, including liver, heart, kidney and skeletal muscle, contain their own intrinsic circadian clocks, as *per* or *bmal1* were found to have a cyclical presence, and were documented to free run in continuous darkness (Yamazaki et al., 2000; Oishi et al., 1998; Whitmore et al., 1998; Zylka et al., 1998). Likewise, *Drosophila, per* was found to be expressed and demonstrate rhythmic activity (ie. cycles in abundance or localization (nuclear vs. cytoplasmic)) in peripheral tissues, including gut, testes, excretory system, alimentary tract, rectum and malphigian tubules (Liu et al., 1988; Saez and Young, 1988; Hege et al., 1997; Plautz et al., 1997; Giebultowicz and Hege, 1997; Gilbultowicz et al., 2001). A new prevalent view developed, one that saw the aforementioned master clock as a synchronizer of peripheral clocks, rather than driving rhythmicity in slave oscillators. In agreement with this, in mammals, the SCN "master" clock neurons were found to have the ability of synchronizing and sustaining each other's rhythms for prolonged periods of time via interneuronal circuit interactions (Nagoshi et al., 2004; Welsh et al., 2004). The fibroblast peripheral clocks drifted out of phase with each other much quicker and the SCN can thus have a role in synchronizing the fibroblast clocks with each other. In the same vein, the same process can be seen in insects. For instance, in *Drosophila,* rhythms in odor sensitivity by the antenna were found to persist in individual olfactory neurons when severed from the optic lobe (Page and Koelling, 2003; Saifullah and Page, 2009). However, the "master" clock, found in the optic lobe, is able to modulate this rhythm and synchronize these individual clocks in the antennae to the overall circadian rhythm of the insect (Saifullah and Page, 2009). Furthermore, PER was found to be expressed rhythmically in antennae, in both olfactory receptor neurons and their supporting cells (Merlin et al., 2006; Schuckel et al., 2007). The sensitivity rhythms in these cells were found to be lost, in *per* null-mutant flies. This further confirms the role and importance of *per* in cellular clockwork. Thus, identification of cycling *per* is a good indicator as to the presence of a potential clock.

3.2 - The requirements for true clock status

In order for a cell to possess an endogenous clock it needs to possess more characteristics rather than just have genes expressed with a daily rhythm. The role of a clock is to be able to temporally synchronize different tissues in an organism with the external environment. A clock should be able to exhibit self-sustaining oscillations *in vitro*. Further, it has to possess the ability to be entrained by environmental signals, such that it can be synchronized with the outside world. Lastly, it has to have rhythmic output, in order to convey this timing information to the rest of the organism. The master clock in mammals and insects meets these criteria, as do those of some peripheral tissues, which thus qualify as proper peripheral clocks (Giebultowicz, 2001). In birds, it was shown that the metalonin synthesis rhythm by the pineal gland and retina persist *in vitro* (Underwood et al., 1997). Further, rhythms in the liver, kidneys and heart of zebrafish were found to persist *in vitro*, as well as being light-entrainable (Whitmore et al., 2000). Also, the aforementioned mammalian fibroblasts (Balsalobre et al, 1998), as well as mammalian liver, lungs and skeletal muscle have true clocks, with rhythms persisting *in vitro* (Yamazaki et al., 2000). In *Drosophila*, rhythms also persist in renal tubules and rectum *in vitro* (Giebultowicz et al., 2000). *Drosophila* legs, proboscis, antennae and wings have oscillations of *per* in cultured conditions, that can be entrained to the external light cycle

(Hege et al., 1997; Plautz et al., 1997; Levine et al., 2002; Glaser and Stanewsky, 2005). These clock gene rhythms are also found to persist after the master clock is removed (Merlin et al., 2007, 2009; Uryu and Tomioka, 2010).

The results of this thesis argue against a local clock in the FB of *Rhodnius,* and conclude that the FB is a driven peripheral oscillator. It should be stressed that though the idea of slave oscillators has fallen out of fashion, in favour of autonomous peripheral oscillators, these two concepts are not mutually exclusive and can co-exist in one organism. It is therefore important to confirm the autonomy of cellular clocks before declaring any peripheral tissue to be a clock.

3.3 - Master clock able to modulate peripheral clocks and oscillators

Evidence exists that the brain clock can modulate peripheral oscillating tissues, affecting core clock genes. In *Drosophila,* the central clock was found to organize temporal structure of the antennae peripheral clock (Page and Koelling, 2003; Saifullah and Page, 2009). In silkworm larvae, it was observed that the rhythmicity of PER in peripheral tissue was disrupted in decapitated animals (Sauman and Reppert, 1998). Also in silkworm larvae, the removal of the optic lobe which houses the master clock was found to affect *per* and *tim* mRNA cycling in peripheral tissues. In the anterior stomach, rhythms of both these clock genes were lost, while in the brain the rhythms persisted, but with a different phase, and in the mid gut and terminal abdominal ganglion *tim* became arrhythmic while *per* kept its rhythm *in vitro* (Uryu and Tomioka, 2010). Thus, control from the master clock in the brain is able to regulate peripheral clocks, even if these clocks can oscillate independently (Uryu and Tomioka, 2010). These data strengthen the case for a hierarchical organization of timekeeping existing in animals as well, while also confirming that rhythmic clock genes such as PER within a tissue is not enough evidence for it being a true clock.

3.4 - Humoral factors provide temporal information: a comparison between the mammalian hypothalamic-pituitary-adrenal (HPA) axis and Rhodnius

In both mammals and insects, peripheral clocks and oscillatorscan be modulated by circulating factors. In mammals, the SCN is known to modulate and regulate the molecular machinery of other cellular clocks and oscillators (reviewed by Albrecht, 2012). It can achieve this via electrical, metabolic or endocrine signals, though the interaction between these pathways and with peripheral clocks and oscillators is not well understood. One system that has been extensively studied in this regard is the HPA axis. The current understanding of the circadian organization of the HPA axis involves light entrainment of the SCN, which subsequently regulates rhythmic secretion of corticotropin-releasing hormone (CRH) and vasopressin (AVP) (reviewed by Son et. al., 2011). CRH and AVP work synergistically to promote synthesis and secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH then acts on adrenocortical cells in the adrenal gland, promoting synthesis and release of glucocorticoids (GCs) (Ishida et. al., 2005; Oster et. al., 2006). Circulating GCs then act on peripheral tissues, including clocks and potential oscillators. Therefore, the mammalian brain clock, the SCN, indirectly provides timing information to clocks, oscillators, and non-clock cells throughout the organism by regulating hormone rhythms.

Balsalobre and colleagues (2000) provided the first line of evidence that GCs are able to phase shift peripheral clocks and oscillators. They showed that GCs are able to phase shift the liver clock, as well as the kidney and heart *in vivo*, as well as in cultured fibroblasts (Balsalobre et. al., 2000). GCs also interact with clock components of the CRY family in adipose tissue, modulating glucose homeostasis (Lamia et al., 2011). Indeed, GCs are found to be important for circadian physiology and metabolism, as they lead to expression of clock genes in peripheral tissues (Balsalobre et al., 2000, Izumo et al., 2006, McNamara et al., 2001). GC presence was found to induce *per1* gene expression in mice peripheral tissues such as liver (Torra et al., 2000, Yamamoto et al., 2005). In fact, *per1* mRNA expression in liver, kidney, and pancreas was found to be related to the GC profile in the blood stream (Son et al., 2008). Indeed, GC-response elements were found to be present in the regulatory regions in the genome of core clock genes such as *bmal1, cry1, and per1* (Reddy et. al., 2007; Yamamoto et. al., 2005) and *per2* (So et. al., 2009). Furthermore, GC was found to have profound effects on core clock gene expression. Chronic administration of GC was found to abolish circadian *per1* mRNA expression, as PER was constantly over-expressed even with an intact molecular oscillator (Koyanagi et al., 2006). Additionally, GC can drive rhythmicity of genes in a more direct fashion, and not through the molecular oscillator (Oishi et al., 2005). In adrenalectomized mice, 100 of 169 rhythmic genes were found to have lost periodic expression, even in the presence of normal cycling core clock genes such as *per2* and Dbox binding protein (DBP) (Oishi et al., 2005). These data conclusively show that the circulating GC rhythm is involved in the entrainment of local peripheral oscillators and clocks, thereby contributes to the maintenance homeostatic balance in mammals.

Although extensive research has been conducted on the HPA axis, it is widely regarded as extremely difficult to map the circadian system in mammals. The simpler model insect *Rhodnius* provides a more tractable system with which to study and elucidate the circadian control of peripheral clocks and oscillators. Further, the *Rhodnius* circadian regulatory system is strikingly similar to the mammalian model. The analogy between the hypothalamo-pituitary system of mammals and the corpus cardiacum-corpus allatum system of insects has been described (Scharrer and Scharrer, 1945). Results from the Steel lab have extended this analogy to include the circadian system (see review by Steel and Vafopoulou, 2006). At the top of the "hierarchy" of circadian organization in *Rhodnius* the brain clock regulates a circadian rhythm of synthesis and secretion of prothoracicotropic hormone (PTTH) (Vafopolou and Steel, 1996b; Vafoopulou et al., 2007; Steel and Vafopoulou, 2006) and there is a known rhythm of responsiveness by the PGs to PTTH (Vafopoulou and Steel, 1999). PTTH acts on the PGs to regulate ecdysteroidogenesis and was found to modulate and set the phase of the intrinsic rhythm of ecdysteroid synthesis by the PGs (Pelc and Steel, 1997). This is much like ACTH peptide from the brain and under the control of the SCN acts on the adrenal clock to produce a rhythm of steroid hormone (i.e. GCs), as described above.Thus, in both the mammalian and insect models, the phase of a peripheral endogenous clock and its output are controlled by the central brain clock. Additionally, much like the rhythmic GC output was found to convey temporal information to peripheral clocks and oscillators throughout the body, and even control and induce expression of core clock genes such as *per1* and *per2* (see above), I have found that rhythmic ecdysteroid output by the PGs induces PER expression by FB in *Rhodnius*, showing that FB is a driven oscillator instead of a self-sustained true clock. Thus, strong parallels can be drawn between the mammalian and insect circadian organizational principles, making insects, especially *Rhodnius*, useful model organisms in which to study these principles. Overall, these results demonstrate remarkable functional and organizational similarities between distantly related phyla and emphasize the vital importance of the circadian system.

3.5 - Why two transcription factors?

Ecdysteroids exert their effects on tissue through an intracellular protein; the ecdysone receptor (EcR). EcR is a transcription factor, and exhibits circadian cycling driven by circulating 20HE, why is it needed for this system to drive PER expression, another transcription factor, with the same phase? To shed some light on this dilemma, it's been found that quite a lot of genes are expressed rhythmically: up to 10% of the genome in peripheral tissues and SCN in mammals (Morse and Sassone-Corsi, 2002). To further complicate things, rhythmic genes are almost always different in different tissues and organs. For instance, when comparing rhythmic genes in heart vs. liver or SCN, only 10% of the cycling transcripts are found in both (Morse and Sassone-Corsi, 2002). Thus, a lot of differential genes have to be driven in each tissue, and this might require more than a single transcription factor, be it controlled by an intrinsic peripheral clock or by exogenous cues. In the mammalian liver, 10-20% of the local transcriptome and 10-20% of the proteome is under circadian regulation (Reddy et al., 2007). As previously mentioned, the liver is a known peripheral clock, however, 90% of the hepatic circadian transcriptome is driven by the local clock (Kornmann et al., 2007). The other 10% of cycling genes are driven by exogenous cues. In *Drosophila* fat body, it was shown that the local oscillations are responsible for 60% of the rhythmic genes, while 40% of them are driven by exogenous factors (Xu et al., 2011). These data suggest a dual role of temporal control and rhythmic gene expression, of both a local oscillator as well as exogenous cues, be it nervous, metabolic, or humoral. Here, this possible model might explain why two transcriptional regulators (EcR and PER) are more efficient than one, in driving rhythmicity in a large number of potential genes.

Another reason for two transcription factors may be that EcR has no specificity for clock genes, and cannot directly drive selective gene expression. Therefore, this could present a mechanism by which the 20HE rhythm can signal cells to transcribe selectively target genes through PER. In other words, two transcription factors could provide a more diverse and intimate control and regulation of target genes in the tissue.

4.6 - Comparison with potential FB peripheral oscillators from other species

Core clock genes have been shown to be expressed and have important roles in both fat body of insects and adipose tissue in mammals, although the evidence is not necessarily conclusive that this organ is a true peripheral clock in insects and *Rhodnius*. In mammals, over-expression of *bmal1in vitro* has been found to have effects on lipid synthesis activity and lipogenesis (Reppert and Weaver, 2002). Also, knocking out *bmal1* via RNAi led to pre-adipocytes failing to differentiate into mature adipocytes or accumulate lipid (Reppert and Weaver, 2002). *Bmal1*, as well as *per1* and *clk* clock genes mRNA were also found to cycle in a circadian fashion in cultured cells of mature white adipose tissue (Shimba et al., 2005). Further, 660 genes, including ones affecting lipid metabolism, are differentially expressed between wild type adipocytes versus *clk* mutant ones (Bray and Young, 2009). These findings are suggestive that core clock components are key regulators of adipogenesis and that energy accumulation by adipose tissue may be time-dependent, and adipose tissue might be a peripheral oscillator. However, all the core clock components were not found to be cycling (Shimba et al., 2005), leading to a doubt if this tissue possesses a proper clock. Also, similar to humoral factors modulating oscillations of the molecular oscillator (MO), here, exogenous factors are most likely modulating adipose tissue oscillations as well, as a change to higher fat diet was found to induce much more elevated levels of *bmal1* mRNA in adipose tissue (Shimba et al., 2005). Since *bmal1* was shown to affect lipid biochemical pathways, this suggests that exogenous factors can communicate, through the clock MO, with adipose tissue, probably in order to modulate its role and function to meet metabolic demands.

In *Drosophila*, a possible clock in fat body was proposed, as clock protein expression in this tissue has been reported (Giebultowicz et al., 2001). *Tim* mRNA was found to cycle under L/D and DD conditions, but not in CLK mutants. Functional CLK expression was knocked out in FB, and this lead to both dampened cycling and decreased levels of *tim* mRNA (Xu et al., 2008). Further, expression of other genes, including metabolic ones, was affected in non-functional CLK mutants. Genes involved in detoxification and immune functions also display circadian rhythms in the FB of *Drosophila* (Krishnan et al., 2008; Lee and Edery, 2008). These can potentially be rhythmically controlled through the MO, or from exogenous factors. The latter is plausible, as restricted feeding was found to drive rhythmic expression of circadian genes, such as *clk*, in the fat body (Xu et al., 2011). Timing of restricted feeding was found to be able to shift rhythmic gene expression. This effect by restricted feeding was not found to occur in the brain, however (Xu et al., 2011). Feeding patterns affect FB oscillations through both CLK dependent and CLK independent pathways, suggesting that this tissue's circadian rhythms and core clock genes can be modulated by exogenous factors, similar to what was found in mammals (Shimba et al., 2005), and my findings in *Rhodnius*. While patterns in feeding were found to affect circadian genes in the FB of *Drosophila*, it is important to note that this could not be the case in *Rhodnius*. This is because *Drosophila* normally feeds every day, mainly during the night (Xu et. al., 2011), while *Rhodnius* does not feed daily, but rather once in an extended period of time, even months. As such, feeding patterns would not be able to affect circadian gene cycling in fat body of *Rhodnius*. However, here, showing that a circadian rhythm of 20HE can affect circadian cycling of PER, this might imply that in *Rhodnius*, the humoral pathway has an increased role in temporally controlling this tissue instead of feeding and dietary cues. Nevertheless, evidence from both the mammalian and insect systems suggests that exogenous factors have significant input to the MO found in the adipose tissue andfat body. This input is found to be controlling the circadian cycling of both core clock genes and others, from immune function and detoxification, to metabolism and energy storage. Thus, even if adipose tissue or fat body of a species is found to not meet all the critera to be considered a true peripheral clock, the core clock components seem to play a vital role in regulating the functions in this tissue, ultimately leading to and providing homeostatic balance in the organism.

REFERENCES

Albrecht Urs. 2012. Timing to perfection: the biology of central and peripheral circadian clocks. *Neuron***74:**246-260.

Balasobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schutz G, Schibler U. 2000. Resetting of circadian time in peripheral tissues by glucocorticoid signalling. *Science***289:**2344-2347.

Balsalobre A, Damiola F, Schibler U. 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell***93:**929–937.

Bray MS, Young ME. 2009. The role of cell-specific circadian clocks in metabolism and disease. *Obes Rev***10:**6-13.

Giebultowicz JM, Hege D. 1997. Circadian clock in malpighian tubules. *Nature***386:**664.

Giebultowicz JM, Stanewsky R, Hall JC, Hege DM. 2000. Transplanted *Drosophila* excretory tubules maintain circadian clock cycling out of phase with the host. *Curr Biol***10:**107-110.

Giebultowicz JM. 2001. Peripheral clocks and their role in circadian timing: insights from insects. *Phil Trans R Soc LondB***356:**1791-1799.

Giebultowicz, JM, Ivanchenko M, Vollintine T. 2001. Organization of the insect circadian system: spatial and developmental expression of clock genes in peripheral tissues of Drosophila melanogaster. In: Denlinger, DL, Giebultowicz JM, Saunders DS (eds)*Insect Timing: Circadian rhythmicity to seasonality*, pp. 31-42.Amsterdam: Elsevier Science.

Glaser FT, Stanewsky R. 2005. Temperature synchronization of the *Drosophila* circadian clock. *Curr Biol***15:**1352-1363.

Hastings MH, O'Neill JS, Maywood, ES. 2007. Circadian clocks: regulators of endocrine and metabolic rhythms. *J Endocrinol***195:**187-198.

Hege DM, Stanewsky R, Hall JC, Giebultowicz JM. 1997. Rhythmic expression of a PER-reporter in the malphigian tubules of decapitated *Drosophila*: evidence for a brain-independent circadian clock. *J Biol Rhythm***12:**300-308.

Ishida A, Mutoh T, Ueyama T, Bando H, Masubuchi S, Nalahara D, Tsujimoto G, Okamura H. 2005. Light activates the adrenal gland: timing of gene expression and glucocorticoid release. *Cell Metab***2:**297-207.

Izumo M, Sato TR, Straume M, Johnson CH. 2006. Quantitative analyses of circadian gene expression in mammalian cell cultures. *PLoS Comput Biol***2:**e136.

Kornmann B, Schaad O, Bujard H, Takahashi JS, Schibler U. 2007. System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. *PLoS Biol***5:**e34.

Koyanagi S, Okazawa S, Kuramoto Y, Ushijima K, Shimeno H, Soeda S, Okamura H, Ohdo S. 2006. Chronic treatment with prednisolone represses the circadian oscillation of clock gene expression in mouse peripheral tissues. *Mol Endocrinol***20:**573-583.

Krishnan N, Davis AJ, Giebultowicz JM. 2008. Circadian regulation of response to oxidative stress in *Drosophila melanogaster*. *Biochem Biophys Res Comm***374:**299–303.

Lamia KA, Papp SJ, Yu RT, Barish GD, Uhlenhaut NH, Jonker JW, Downes M, Evans RM. 2011. Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature***480:**552-556.

Lee JE, Edery I. 2008. Circadian regulation in the ability of Drosophila to combat pathogenic infections.*Curr Biol***18:**195–199.

Levine JD, Funes P, Dowse HB, Hall JC. 2002. Advanced analysis of a *cryptochrome* mutation's effects on the robustness and phase of molecular cycles in isolated peripheral tissues of *Drosophila*. *BMC Neurosci***3:**5.

Liu X, Lorenz L, Yu Q, Hall JC, Rosbash M. 1988. Spatial and temporal expression of the *period* gene in *Drosophila melanogaster*. *Genes Dev***2:**228-238.

McNamara P, Seo SB, Rudic RD, Sehgal A, Chakravarti D, FitzGerald GA. 2001. Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. *Cell***105:**877-889.

Merlin C, Francois MC, Queguiner I, Maibeche-Coisne M, Jacquin-Joly E. 2006. Evidence for a putative antennal clock in *Mamestra brassicae*: molecular cloning and characterization of two clock genes – *period* and *cryptochrome* – in antennae. *Insect Mol Biol***15:**137-145.

Merlin C, Gegear RJ, Reppert SM. 2009. Antennal circadian clocks coordinate sun compass orientation in migratory monarch butterflies. *Science* **325:** 1700-1704.

Merlin C, Lucas P, Rochat D, Francois MC, Maibeche-Coisne M and Jacquin-Joly E. 2007. An antennal circadian clock and circadian rhythms in peripheral pheromone reception in the moth *Spodoptera littoralis*. *J Biol Rhythm* **22:** 502-514.

Morse D, Sassone-Corsi P. 2002. Time after time: inputs to and outputs from the mammalian circadian oscillators. *Trend Neurosci* **25(12):** 632-637.

Nagoshi E, Saini C, Bauer C, Laroche T, Naef F, Schibler U. 2004. Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* **119:** 693–705.

Oishi K, Sakamoto K, Okada T, Nagase T, Ishida N. 1998. Antiphase circadian expression between BMAL1 and *period* homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats. *Biochem Biophys Res Comm* **253:** 199-203.

Oishi M, Suwazini Y, Sakata K, Okubo Y, Harada H, Kobayashi E, Uetani M, Nogawa K. 2005. A longitudinal study on the relationship between shift work and the progression of hypertension in male Japanese workers. *J Hypertens* **23:** 2173-2178.

Oster H, Damerow S, Kiessling S, Jakubcakova V, Abraham D, Toam J, Hoffmann MW, Eichele, G. 2006. The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal clock. *Cell Metab* **4:** 163-173.

Page TL, Koelling E. 2003. Circadian rhythm in olfactory response in the antennae controlled by the optic lobe in the cockroach. *J Insect Physiol* **49:** 697-707.

Pelc D, Steel CGH. 1997. Rhythmic steroidogenesis by the prothoracic glands of the insect *Rhodnius prolixus* in the absence of rhythmic neuropeptide input: implications for the role of prothoracicotropic hormone. *Gen Comp Endocrinol* **108(3):** 358-365.

Pittendrigh CS. 1974. Circadian oscillations in cells and the circadian organization of multicellular systems. In: Schmitt FO, Worden GO (eds) *The Neurosciences: Third Study Program,* pp. 437-458. Cambridge: MIT Press.

Plautz JD, Kaneko M, Hall JC, Kay SA. 1997. Independent photoreceptive circadian clocks throughout *Drosophila*. *Science* **278:** 1632-1635.

Reddy AB, Maywood ES, Karp NA, King VM, Inoue Y, Gonzales FJ, Lilley KS, Kyriacou CP, Hastings MH. 2007. Glucocorticoid signaling synchronizes the liver circadian transcriptome. *Hepat* **45:** 1478-1488.

Reppert SM, Weaver DR. 2002. Coordination of circadian timing in mammals. *Nature***418:**935–941.

Saez L, Young MW. 1988. *In situ* localization of the *per* clock protein during development of *Drosophila melanogaster*. *Mol Cell Bio***8:**5378-5385.

Saifullah ASM, Page TL. 2009. Circadian regulation of olfactory receptor neurons in the cockroach antenna. *J Biol Rhythm***24:**144-152.

Sauman I, Reppert SM. 1998. Brain control of embryonic circadian rhythms in the silkworm, *Antheraea pernyi*. *Neuron***20:**741-748.

Scharrer E, Scharrer B. 1945. Neurosecretion. *Physiol Rev***25:**171-181.

Schuckel J, Siwicki KK, Stengl M. 2007. Putative circadian pacemaker cells in the antenna of the hawkmoth *Manduca sexta*. *Cell Tissue Res***330:**271-278.

Shimba S, Ishii N, Ohta Y, Ohno T, Watabe Y, Hayashi M, Wada T, Aoyagi T, Tezuka M. 2005. Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. *Proc Nat Acad Sci USA***102(34):**12071-12076.

So AY, Bernal TU, Pillsbury ML, Yamamoto KR, Feldman BJ. 2009. Glucocorticoid regulation of the circadian clock modulates glucose homeostasis. *Proc Nat Acad Sci USA***106:**17582-17587.

Son G H, Chung S, Kim K. 2011. The adrenal peripheral clock: glucocordicoid and the circadian timing system. *Front Neuroendocrinol***32:**451-465.

Son GH, Chung S, Choe HK, Kim HD, Baik SM, Lee H, Lee HW, Choi S, Sun W, Kim H, Cho S, Lee KH and Kim K. 2008. Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. *Proc Nat Acad Sci USA***105:**20970-20975.

Steel CGH, Vafopoulou X. 2006. Circadian orchestration of developmental hormones in the insect, *Rhodnius prolixus*. *Comp Biochem Physiol A***144:**351-364.

Tomioka K, Abdelsalam S. 2004. Circadian organization in hemimetobolous insects. *Zool Sci***21(12):**1153-1162.

Torra IP, Tsibulsky V, Delaunay F, Saladin R, Laudet V, Fruchart JC, Kosykh V, Staels B. 2000. Circadian and glucocorticoid regulation of Rev-erbalpha expression in liver. *Endocrinol***141:**3799-3806.

Underwood HA, Wassmer GT, Page TL. 1997. Daily and seasonal rhythms. In: Dantzler WH (ed) *Handbook of Physiology,* pp. 1653-1763. New York: Oxford University Press.

Uryu O, Tomioka K. 2010 Circadian oscillations outside the optic lobe in the cricket *Gryllus bimaculatus*. *J Insect Physiol***56:**1284-1290.

Vafopoulou X, Steel CGH. 1996a. The insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: re-evaluation of its role in development. *Proc Nat Acad Sci USA***93(8):**3368-3372.

Vafopoulou X, Steel CGH. 1996b. Circadian regulation of a daily rhythm of release of prothoracicotropic hormone from the brain-retrocerebral complex of *Rhodnius prolixus* (Hemiptera) during larval-adult development. *Gen Comp Endocrinol***102(1):**123-129.

Vafopoulou X, Steel CGH. 1999. Daily rhythm of responsiveness to prothoracicotropic hormone in prothoracic glands of *Rhodnius prolixus*. *Arch Insect Biochem Physiol***41:**117-123.

Vafopoulou X, Steel CGH, Terry KL. 2007. Neuroanatomical relations of prothoracicotropic hormone neurons with the circadian timekeeping system in the brain of larval and adult *Rhodnius prolixus* (Hemiptera). *J Comp Neurol***503(4):**511-524.

Welsh DK, Yoo SH, Liu AC, Takahashi JS, Kay SA. 2004. Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Curr Biol***14:**2289–2295.

Whitmore D, Foulkes NS, Sassone-Corsi. 2000. Light acts directly on organs and cells in culture to set the vertebrate circadian clock. *Nature***404:**87-91.

Whitmore D, Foulkes NS, Strahle U, Sassone-Corsi P. 1998. Zebrafish *Clock* rhythmic expression reveals independent peripheral circadian oscillators. *Nature Neurosci***1:**701-708.

Xu K, DiAngelo JR, Hughes ME, Hogenesch JB, Sehgal A. 2011. Interaction between circadian clocks and metabolic physiology: implications for reproductive fitness. *Cell Metab***13(6):**639-654.

Xu K, Zheng X, Sehgal A. 2008. Regulation of feeding and metabolism by neuronal and peripheral clocks in *Drosophila*. *Cell Metab***8(4):**289-300.

Yamamoto T, Nakahata Y, Tanaka M, Yoshida M, Soma H, Shinohara K, Yasuda A, Mamine T, Takumi T. 2005. Acute physical stress elevates mouse period1 mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. *J Biol Chem* **280:**42036-42043.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, Tei H. 2000. Resetting central and peripheral circadian oscillators in transgenic rats. *Science* **288:**682–685.

Zylka MJ, Shearman LP, Weaver DR, Reppert SM. 1998. Three *period* homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron* **20:**1103-1110.

APPENDIX

SD = Standard Deviation; SEM = Standard Error of the Mean

Figure 2. Day 12 1h after lights off

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
89.11	57.491	180.349	218.996
99.242	74.986	196.566	214.018
121.102	51.999	141.514	201.322
114.203	48.09	209.079	188.665
83.519	55.537	206.327	212.368
112.672	59.342	181.229	206.955
82.547	44.259	187.82	163.896
113.939	52.413	201.448	153.432
86.413	39.328	195.107	159.076
75.958	58.118	126.252	153.015
72.823	79.496	102.557	167.137
87.772	99.208	115.036	154.131
76.905	35.767	196.501	144.347
99.535	70.142	210.604	162.22
94.519	55.732	220.031	178.4
71.559	47.933	202.99	163.86
99.971	53.561	213.545	181.776
72.54	71.527	216.188	161.206
89.572	62.957	158.813	172.764
82.927	59.695	177.47	164.12
52.926	35.837	161.708	165.909
124.854	40.97	126.073	129.181
89.902	48.438	151.332	158.296
63.382	39.195	138.642	131.19
66.965	75.694	115.724	147.464
89.556	103.039	107.59	142.251
65.046	101.591	120.819	168.111
68.088	110.003	124.38	145.455
57.493	75.326	121.837	95.898
34.825	66.093	154.57	101.57
37.339	68.461	108.754	91.688
55.68	65.961	148.391	135.822

54.553	97.118	192.142	139.835
64.395	92.861	131.423	127.317
32.502	60.096	131.713	141.881
56.557	76.19	127.636	126.422
74.842	68.309	118.305	144.534
71.127	57.647	193.692	133.612
78.575	90.331	174.838	143.839
50.834	63.58	154.651	119.356
48.081	56.624	128.751	137.95
40.405	73.739	105.928	149.599
60.437	94.076	133.2	135.875
85.375	46.148	140.714	167.42
59.472	80.77	165.612	170.434
46.865	66.898	193.476	170.617
55.844	104.501	169.36	171.479
74.487	64.065	201.569	152.323
66.652	41.055	158.883	150.186
44.401	52.645	107.087	155.837
55.771	79.543	117.668	153.524
47.264	62.098	119.925	152.677
40.04	79.907	182.576	167.218
38.098	76.51	182.737	177.147
67.18	79.626	164.812	173.361
64.632	90.719	185.175	200.444
81.354	98.673	160.217	194.215
81.579	85.377	174.157	202.201
91.836	72.767	181.244	227.816
48.71	64.259	179.776	211.7
40.429	56.148	150.428	208.776
46.766	58.318	128.701	195.23
30.613	40.341	112.539	194.807
47.908	41.254	142.114	196.197
55.952	85.532	166.687	177.937
48.395	38.779	234.838	185.861
57.223	51.386	239.407	177.74
71.318	61.941	230.295	176.55
37.664	71.915	239.443	173.697
53.436	56.602	226.445	155.856
38.134	97.242	222.016	168.793
38.373	86.124	224.855	234.981

	76.167	107.463	211.791	207.394
	67.643	85.339	212.508	231.281
	47.208	43.184	219.161	215.51
Mean		**67.586**		**167.278**
SD		**21.012**		**35.131**
SEM		**1.716**		**2.869**

Figure 2. Day 12 7h after lights off

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
52.722	45.469	116.33	180.784
59.185	36.549	119.896	183.107
71.292	29.565	116.28	160.92
59.217	65.702	144.183	164.707
63.112	65.099	145.422	144.62
45.532	46.54	135.56	164.769
44.986	60.947	143.805	177.49
53.675	58.51	122.377	125.842
71.992	73.124	135.311	126.546
71.243	68.471	158.65	114.45
73.607	81.997	142.945	159.508
52.116	60.317	144.395	170.447
50.395	51.73	130.306	186.858
37.463	64.762	106.831	71.672
42.812	67.295	121.632	59.238
42.458	70.671	148.298	57.143
52.557	55.491	104.233	168.894
66.164	59.107	149.251	119.327
34.786	47.567	128.691	115.582
126.602	42.822	99.628	165.215
65.734	68.568	108.455	175.26
42.861	82.714	152.723	161.278
30.53	51.96	141.64	93.344
58.649	55.678	161.442	115.439
42.242	50.735	136.131	84.473
58.524	48.829	126.696	117.218
32.933	50.961	91.571	90.381
31.99	49.076	85.38	121.52
47.971	55.627	74.58	190.93
65.14	89.28	103.248	180.576

60.014	73.697	79.863	164.926
47.778	81.277	78.285	132.341
55.276	76.306	60.382	130.252
78.875	50.287	86.114	107.116
27.998	55.156	67.98	174.321
28.941	60.029	89.986	157.998
46.233	48.011	62.738	186.51
48.541	57.472	77.218	161.712
66.205	62.091	132.41	160.513
67.792	55.619	117.87	161.247
49.409	62.865	114.498	164.957
66.864	54.798	136.967	204.791
71.543	68.137	170.974	163.267
48.808	51.04	115.574	164.318
146.306	56.759	132.567	160.234
78.878	69.087	178.54	189.385
101.413	85.494	113.588	160.445
62.52	75.777	135.793	150.735
68.684	70.454	144.77	155.958
68.012	80.839	168.252	136.933
62.636	67.62	118.755	142.151
41.515	71.669	98.984	130.754
53.63	87.906	114.899	144.924
94.765	53.286	118.987	134.442
72.425	45.376	131.993	133.77
44.696	45.565	140.507	105.54
46.69	48.24	148.128	131.841
47.542	62.69	129.342	154.738
46.177	46.19	129.27	158.802
47.83	52.872	100.708	155.204
55.855	40.979	110.436	166.329
45.246	70.833	103.781	164.751
52.055	76.296	128.137	140.291
58.696	73.093	96.74	202.34
96.158	68.657	53.628	168.373
44.933	47.791	49.395	191.964
48.234	81.903	43.79	140.213
87.996	105.029	82.142	179.494
47.909	77.254	62.893	163.699
128.351	81.584	91.311	144.767

	84.659	103.303	118.671	144.759
	56.967	102.816	101.66	166.44
	60.269	88.768	102.944	161.566
	61.339	55.063	201.496	170.352
	55.798	101.216	193.422	197.609
Mean		**61.915**		**133.753**
SD		**19.187**		**35.748**
SEM		**1.567**		**2.919**

Figure 2. Day 12 1h after lights on

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
30.645	42.171	38.338	112.69
41.407	36.923	43.924	101.851
87.107	54.258	43.35	117.667
72.908	56.224	84.587	106.403
82.97	55.343	71.31	112.898
93.63	56.586	77.312	116.146
87.686	35.551	73.707	128.769
54.02	25.906	64.38	87.456
62.146	74.368	78.531	88.298
58.75	35.24	67.325	80.692
59.717	44.939	56.638	112.957
65.847	35.131	62.848	122.832
49.227	38.046	44.808	115.231
72.131	18.96	41.464	191.167
43.265	34.678	36.213	139.169
44.941	22.195	37.82	170.119
47.515	31.514	37.169	149.373
34.022	66.989	32.81	142.66
18.688	75.604	38.915	156.539
34.645	78.53	66.692	137.261
20.029	52.885	69.352	96.483
15.333	81.664	65.379	113.946
20.918	48.177	70.103	81.587
37.442	50.621	54.599	89.822
70.637	57.989	47.296	72.534
73.529	48.282	45.63	91.55
113.245	61.901	46.986	91.158

86.696	47.393	51.143	113.316
41.223	32.753	51.476	102.67
70.244	64.122	55.51	97.761
42.091	56.171	37.537	81.622
62.15	59.48	33.225	72.895
58.754	42.033	38.609	63.374
31.986	110.338	59.844	80.529
55.135	64.063	67.591	89.642
33.187	60.871	75.244	67.277
37.166	70.174	71.876	65.795
41.961	33.339	73.257	52.184
35.265	31.085	71.45	77.83
47.615	43.239	80.165	45.594
69.696	41.279	48.883	76.827
34.562	48.505	55.24	60.186
54.868	44.7	71.18	72.814
41.953	40.124	56.77	65.345
40.032	36.312	52.287	68.609
38.34	29.348	85.479	60.508
44.235	30.516	81.885	63.378
30.808	36.146	70.289	81.56
36.552	31.676	84.166	76.176
43.619	25.07	88.892	43.208
42.415	35.408	59.324	50.11
33.666	27.796	46.722	58.52
35.971	23.091	46.914	52.554
34.359	48.18	42.28	49.201
41.43	42.525	49.881	55.693
38.22	46.68	51.49	74.715
78.519	60.019	42.21	216.272
49.345	64.398	129.201	175.501
59.097	36.108	128.673	213.291
55.646	29.552	133.226	164.64
42.571	32.747	147.213	139.55
37.401	29.043	104.238	154.56
48.2	28.943	89.63	64.225
50.685	32.113	101.436	55.883
46.341	43.182	128.379	56.794
45.485	35.901	115.844	136.483
41.959	29.999	131.337	97.198

	65.747	25.279	140.899	86.27
	53.472	71.54	139.562	116.365
	52.359	56.826	156.117	169.586
	48.452	44.621	144.235	201.391
	42.326	46.334	114.206	154.34
	38.173	43.339	135.794	160.65
	44.658	35.685	116.703	130.691
	42.485	37.009	113.189	143.468
Mean		**47.635**		**89.017**
SD		**17.582**		**40.81**
SEM		**1.436**		**3.332**

Figure 2. Day 12 7h after lights on

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
38.954	28.501	67.964	50.679
33.9	31.512	67.104	44.79
43.072	50.206	109.595	40.536
26.007	40.721	65.924	37.803
30.658	32.808	89.423	78.213
25.711	41.483	90.273	70.735
46.118	31.465	108.92	61.624
28.013	27.012	131.563	66.501
48.823	32.807	110.286	72.907
33.049	40.415	45.476	62.835
39.492	52.714	46.173	80.311
25.988	44.313	43.271	69.552
29.601	50.837	39.709	68.273
29.391	87.567	44.741	96.491
56.35	50.357	39.75	87.789
45.361	59.297	155.692	88.982
45.196	53.679	123.253	74.72
32.487	51.34	153.37	82.176
52.593	93.319	108.152	57.29
41.332	56.075	123.765	61.98
18.754	43.748	124.888	52.615
58.425	40.919	87.326	58.564
43.641	37.988	68.58	57.56
57.045	33.466	94.742	42.745
59.373	30.151	78.886	79.94

55.355	31.616	155.897	91.952
104.757	45.363	170.482	68.271
100.609	38.55	135.362	62.439
93.666	51.65	121.778	53.474
56.066	31.863	95.772	47.883
49.397	37.149	96.645	34.382
81.021	35.8	141.701	42.808
67.85	24.899	140.387	30.774
77.897	44.521	147.15	72.346
42.783	40.68	90.231	93.249
96.14	54.223	89.289	84.141
62.671	42.111	78.378	78.109
72.794	38.39	85.59	70.385
54.243	35.453	74.512	83.993
100.957	26.674	70.976	72.442
101.059	70.081	84.487	74.532
71.933	35.221	76.814	68.448
89.227	41.232	87.286	106.438
52.221	64.392	143.443	99.246
84.215	56.351	116.406	79.173
71.18	61.376	114.197	79.821
79.096	60.271	88.243	66.846
69.815	50.815	92.124	71.32
73.988	51.429	78.887	74.851
43.198	47.906	66.781	79.938
71.918	40.631	63.958	57.566
71.338	33.385	66.265	89.39
79.902	27.014	54.24	64.762
67.639	38.812	65.54	86.839
80.57	34.213	50.483	71.819
79.191	43.652	56.701	70.638
74.34	38.68	62.963	45.175
96.337	39.16	86.524	53.52
84.717	46.34	68.61	46.408
94.339	69.306	65.463	43.494
73.353	31.029	52.683	48.793
53.203	28.14	50.316	41.534
67.256	48.418	37.542	43.19
46.819	62.445	44.633	51.197
69.068	57.143	78.851	53.32

	70.641	87.156	61.465	54.616
	53.281	69.554	59.256	78.995
	70.605	86.871	81.757	76.672
	73.096	106.886	67.354	70.721
	56.27	21.75	74.747	55.906
	53.897	43.762	80.136	53.541
	34.263	21.032	62.886	41.39
	29.767	45.583	60.608	51.274
	45.724	42.867	69.143	47.448
	32.791	34.65	68.716	44.287
Mean		**52.873**		**75.519**
SD		**20.684**		**27.856**
SEM		**1.689**		**2.275**

Figure 2. Day 13 1h after lights off

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
53.564	70.735	106.124	146.135
37.205	71.653	138.346	194.503
31.319	65.096	72.668	186.308
32.4	50.566	73.84	178.192
44.169	85.546	87.981	168.577
37.971	147.891	72.282	209.152
60.222	96.517	108.753	173.421
64.507	115.017	102.398	167.459
36.29	69.765	88.897	186.992
24.724	53.099	107.561	162.886
30.097	94.707	99.97	166.61
30.336	79.309	46.743	180.188
57.469	39.707	134.725	187.478
34.842	68.851	48.245	195.881
32.225	85.288	120.785	200.569
38.421	49.531	126.596	197.385
24.503	37.234	82.305	175.937
23.209	71.172	89.335	233.425
28.838	60.597	42.269	227.164
37.746	79.857	51.824	238.406
51.536	65.292	44.87	180.467
36.347	71.519	102.523	172.882
34.253	65.352	59.679	137.576

21.644	55.244	71.922	111.431
40.07	58.767	79.109	130.982
45.198	57.789	118.461	131.944
27.504	71.025	45.858	178.782
27.795	59.313	66.348	225.379
20.838	79.964	87.288	219.963
26.993	65.545	104.126	180.729
43.595	145.751	82.891	193.962
22.15	84.094	67.729	169.728
30.312	110.298	90.857	209.908
40.159	111.409	75.227	201.412
39.465	107.03	80.351	238.799
37.113	150.909	75.266	247.284
37.466	150.704	168.525	205.346
29.297	143.589	194.473	218.493
35.514	116.777	213.872	140.118
47.981	95.496	217.303	162.637
49.918	135.078	135.228	151.925
33.445	146.997	223.246	159.487
50.303	90.507	196.883	164.449
35.544	124.907	191.239	168.297
28.317	121.281	218.26	164.315
29.783	131.634	153.996	182.325
33.367	132.394	128.763	115.428
36.068	90.389	146.846	122.581
34.721	89.03	152.89	115.202
33.707	107.654	170.532	145.553
41.568	78.192	126.451	137.738
136.467	125.713	141.928	135.827
137.53	120.069	174.64	129.408
127.576	159.65	154.125	129.74
138.177	188.197	166.145	135.602
158.808	163.742	206.491	102.719
126.303	142.877	148.253	108.726
83.492	138.604	138.592	176.955
104.711	121.279	155.801	163.481
85.636	52.336	169.508	155.409
118.718	93.705	122.515	166.352
76.822	122.112	145.774	174.156
76.282	63.744	129.812	148.38

	64.171	67.615	153.687	143.872
	59.972	81.531	163.215	140.595
	59.555	68.653	140.47	169.151
	98.607	85.213	163.394	175.106
	101.439	82.487	119.291	127.32
	84.297	86.682	141.27	55.285
	98.68	84.194	124.963	59.14
	102.717	78.766	116.735	40.852
	98.241	71.519	113.892	39.613
	90.981	76.068	103.624	35.532
	67.13	92.081	140.639	124.94
	66.547	70.71	121.221	94.54
Mean		**75.123**		**141.599**
SD		**38.609**		**49.471**
SEM		**3.153**		**4.039**

Figure 2. Day 13 7h after lights off

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
52.594	107.934	252.635	97.231
63.97	124.885	248.33	102.487
45.601	92.707	248.577	108.25
42.952	92.068	226.432	182.332
41.215	103.984	232.29	135.889
61.529	109.291	245.401	142.132
54.995	120.866	131.276	137.28
55.94	78.383	164.79	165.234
56.421	88.171	135.605	147.701
62.855	106.742	175.194	149.985
57.022	89.688	184.812	141.615
70.267	117.281	182.264	100.355
65.868	127.164	141.65	101.329
52.022	62.006	174.878	102.527
71.745	104.133	179.266	103.476
53.698	89.858	239.426	103.258
64.135	74.909	158.541	105.834
39.294	111.576	223.516	136.244
59.551	121.818	211.856	133.447
70.084	96.172	182.31	106.902
48.305	85.606	229.265	169.918

45.082	126.089	243.189	145.837
61.088	108.169	244.42	158.872
59.386	63.459	242.287	116.131
43.543	85.819	124.926	180.145
34.662	53.015	133.581	161.113
56.835	59.237	123.91	150.857
36.136	54.458	173.792	201.261
59.833	62.37	150.756	208.873
66.515	46.751	147.726	138.55
65.536	83.979	207.183	164.637
61.712	42.695	234.864	151.152
48.431	65.406	224.147	157.928
38.505	82.975	121.795	158.872
47.227	85.767	117.716	176.529
62.775	85.726	111.735	205.166
50.051	77.826	238.985	159.716
74.065	58.221	246.647	170.559
60.213	62.026	237.268	157.973
69.977	60.862	209.304	187.58
27.842	49.222	200.66	194.851
53.466	67.898	220.874	190.581
70.961	75.48	222.404	201.127
76.911	76.755	217.651	203.237
52.543	112.672	231.153	160.777
52.968	74.63	169.694	180.921
110.509	81.374	133.284	144.156
134.481	68.261	168.626	121.892
99.434	66.426	124.706	166.377
132.213	70.997	131.479	111.147
168.756	87.525	136.561	102.321
125.016	74.142	167.742	128.53
143.611	89.058	143.57	118.519
155.99	82.968	108.375	82.82
125.391	52.376	119.664	122.942
175.842	71.391	118.515	108.875
145.736	67.342	107.643	103.261
101.052	85.44	117.905	131.503
128.57	77.817	128.914	123.706
113.216	83.897	140.17	105.857
142.46	87.203	150.784	131.549

	140.289	68.977	122.926	107.872
	135.45	74.833	99.882	123.626
	156.722	75.24	159.885	89.65
	141.309	64.803	154.761	128.991
	113.876	161.382	136.61	98.885
	104.85	150.141	106.263	99.223
	127.527	105.989	112.706	144.235
	146.131	137.663	97.817	107.509
	126.271	134.361	110.157	125.527
	132.32	124.754	106.372	169.542
	162.486	132.12	116.254	188.28
	171.986	86.997	71.277	210.452
	159.003	140.106	82.225	155.456
	131.291	189.635	65.63	190.568
Mean		**88.254**		**154.704**
SD		**36.055**		**45.409**
SEM		**2.944**		**3.708**

Figure 2. Day 13 1h after lights on

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
38.955	22.544	131.118	51.486
40.966	21.875	85.181	41.65
46.317	25.834	98.831	143.964
82.917	18.525	117.608	129.655
54.531	20.774	102.544	140.871
61.479	20.458	93.653	109.313
62.397	27.98	55.758	101.504
53.021	25.315	54.321	67.192
34.803	32.841	36.561	80.864
41.745	14.411	125.332	60.362
72.302	35.261	126.268	61.605
59.138	32.089	126.525	108.445
45.336	64.428	106.541	118.692
46.435	51.374	132.486	177.763
60.754	49.843	94.379	144.76
47.82	47.246	134.17	90.173
63.859	50.735	103.37	151.772
40.494	41.802	147.792	56.352
46.146	68.839	124.672	59.776

58.174	66.763	132.318	82.208
56.904	46.181	118.567	135.524
43.448	58.293	179.847	101.212
41.181	45.628	176.919	68.244
42.867	50.479	118.299	88.63
58.173	48.583	145.132	83.255
51.776	37.296	87.72	96.39
49.635	53.818	81.552	103.862
49.164	55.301	131.762	92.457
36.375	72.348	120.57	44.826
58.465	63.408	127.979	66.705
58.556	50.811	103.766	53.92
59.453	50.449	93.342	82.825
25.536	39.735	74.872	81.714
16.379	66.063	95.447	73.484
14.179	42.072	118.243	77.827
16.74	62.563	137.458	65.237
19.276	49.086	144.787	81.341
19.037	70.969	100.862	87.711
34.126	49.373	66.375	81.135
25.958	36.953	98.138	101.789
29.202	59.836	113.487	84.274
15.092	54.815	110.118	65.81
22.405	53.23	107.598	76.419
26.673	34.668	80.24	56.68
23.605	52.746	60.465	63.172
16.771	40.668	82.193	67.863
29.169	37.67	76.834	116.957
21.289	46.284	98.431	102.468
15.385	26.917	62.302	114.745
24.619	47.273	106.236	118.271
16.25	39.703	65.639	142.859
27.612	35.73	80.802	121.776
17.074	42.435	43.934	71.186
19.928	40.07	39.347	81.652
30.227	32.148	32.55	119.39
21.391	24.823	48.171	94.251
24.412	27.936	53.311	84.208
30.495	24.257	37.86	86.202
25.894	25.393	42.159	26.195

	33.692	28.38	21.829	29.872
	28.538	45.667	18.635	32.298
	20.992	53.109	20.233	100.12
	21.454	51.611	19.855	93.113
	29.639	52.314	20.516	49.201
	23.458	38.142	13.687	58.72
	35.416	37.749	59.771	43.111
	19.997	45.853	17.84	33.544
	27.847	60.068	36.149	43.682
	18.463	39.836	36.776	36.616
	24.439	35.268	59.328	53.854
	31.104	35.624	30.218	34.852
	17.119	41.643	43.476	71.25
	20.281	49.235	20.269	47.71
	22.332	47.142	30.587	53.649
	25.861	71.848	50.73	52.603
Mean		**39.609**		**83.118**
SD		**15.672**		**37.399**
SEM		**1.28**		**3.054**

Figure 2. Day 13 7h after lights on

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
44.623	8.204	87.43	94.988
50.01	9.39	77.287	88.563
40.407	12.914	119.27	99.954
49.219	15.428	85.395	104.871
56.706	14.739	86.455	92.32
55.007	16.621	75.643	134.746
36.781	18.637	96.174	120.558
42.817	20.866	131.726	97.93
58.218	17.188	79.236	108.214
70.767	10.466	144.759	95.758
42.285	17.915	89.762	95.101
38.006	23.639	111.858	156.138
24.047	14.691	117.978	101.808
34.724	20.06	75.855	108.155
37.476	34.51	80.531	129.141
45.51	38.17	107.872	130.63
35.629	51.717	125.161	159.735

60.621	33.629	138.333	169.402
31.643	35.052	69.328	104.31
52.823	33.099	58.314	99.414
27.442	25.146	73.524	114.639
35.147	20.755	85.959	126.389
30.356	22.593	73.614	98.773
28.632	36.905	88.758	149.982
55.941	61.343	91.217	133.98
43.123	53.345	105.664	89.323
46.857	43.659	113.488	88.97
47.511	48.576	64.192	87.615
55.83	34.192	68.729	96.508
40.852	36.29	60.861	100.903
47.032	67.25	106.591	84.361
45.966	37.931	90.748	125.852
44.654	52.543	80.556	90.715
28.767	36.974	63.782	131.94
41.267	28.655	39.199	171.449
40.489	44.384	72.582	173.218
26.708	48.607	64.14	82.593
16.041	79.937	64.724	80.964
33.667	130.003	67.195	115.337
34.698	73.036	126.873	146.466
36.562	84.227	132.919	94.992
28.692	61.595	124.311	73.194
49.412	86.999	35.606	108.511
36.78	75.068	30.902	82.872
40.122	65.349	22.815	105.22
33.284	74.324	34.724	118.738
47.057	114.013	23.13	122.584
37.271	105.683	28.476	139.572
19.738	61.353	32.522	148.881
14.775	80.098	32.147	141.122
25.501	91.502	41.239	151.763
20.7	62.841	39.585	152.47
15.301	67.032	39.165	151.582
16.819	89.498	34.437	167.358
18.861	61.967	19.363	169.426
20.807	70.892	31.877	140.677
12.947	50.566	97.193	143.263

	28.159	80.089	123.867	111.52
	24.02	54.086	127.23	73.283
	22.01	47.89	126.501	103.917
	19.946	51.794	121.592	100.761
	18.673	39.073	116.235	90.388
	14.36	54.256	110.518	93.46
	15.372	62.802	128.801	52.585
	17.581	41.771	91.628	70.498
	12.617	40.381	124.385	55.223
	9.107	78.098	154.847	127.328
	13.207	102.247	124.154	100.798
	15.953	68.454	120.877	110.54
	9.75	97.279	177.66	58.62
	21.237	101.548	178.202	55.326
	16.885	54.267	203.583	47.374
	11.453	53.635	128.259	77.544
	13.605	60.833	98.485	41.701
	11.078	50.633	144.85	48.87
Mean		**42.128**		**99.843**
SD		**24.058**		**37.778**
SEM		**1.964**		**3.085**

Figure 3. Day 11 1h in subjective night

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
39.771	149.384	79.499	178.716
32.256	119.078	145.373	156.77
50.535	132.625	131.833	173.72
30.901	103.742	140.954	149.057
44.654	139.277	124.471	172.832
25.48	88.04	111.714	195.852
33.296	170.32	161.849	214.23
30.925	200.101	122.249	166.289
42.824	138.468	195.052	154.573
25.885	132.016	198.584	175.047
29.899	139.961	102.24	156.967
33.39	132.911	129.434	152.956
38.409	138.698	131.708	144.857
41.081	128.126	98.397	152.914
46.079	131.864	128.922	129.09

52.614	121.832	160.493	144.891
51.622	136.487	137.246	165.836
66.98	158.465	187.894	149.754
30.91	82.635	102.165	171.936
53.413	96.002	122.305	127.025
31.257	127.848	107.04	136.743
24.554	119.324	149.771	192.974
50.99	154.528	150.574	158.551
49.289	99.846	134.833	186.931
48.804	86.646	113.117	242.763
40.084	49.14	133.211	228.333
63.559	99.01	93.977	240.249
52.419	72.793	132.182	217.278
54.878	69.011	114.344	226.732
31.548	114.706	112.358	227.34
49.799	96.213	150.184	203.394
49.515	112.808	170.1	206.138
69.56	69.809	122.345	180.217
68.17	73.422	142.308	227.216
114.857	128.974	157.088	205.323
75.692	147.271	174.783	189.597
93.149	80.15	152.42	214.796
65.621	129.284	102.593	221.18
77.468	91.29	115.56	216.155
93.681	93.724	110.198	196.206
145.458	92.446	141.437	220.093
108.166	135.077	118.096	190.387
47.548	125.579	165.157	207.956
91.398	72.659	139.078	189.79
103.811	92.009	173.437	204.168
128.775	75.6	128.78	213.887
99.74	98.288	138.698	221.71
97.343	106.334	129.391	218.747
110.541	73.346	214.731	232.116
86.477	100.204	225.01	123.855
92.994	92.863	225.543	211.777
120.645	129.819	210.663	192.346
104.667	69.497	208.394	153.072
74.693	57.393	218.61	197.761
98.819	28.879	185.533	188.597

	82.052	35.153	169.51	193.272
	133.126	42.03	182.117	188.543
	105.104	38.167	228.869	191.287
	125.989	53.527	220.153	202.886
	129.128	49.523	174.998	189.147
	124.671	46.352	181.323	245.182
	129.567	60.525	203.09	233.625
	105.459	49.268	165.194	244.719
	139.51	54.907	198.963	210.646
	119.546	47.93	228.284	236.46
	115.394	43.771	232.318	233.836
	104.139	52.077	219.281	203.65
	50.95	146.061	208.732	143.749
	47.243	158.19	221.821	196.647
	64.437	146.047	192.322	250.855
	56.589	186.638	184.697	250.624
	53.202	132.04	164.725	242.352
	150.536	86.356	154.384	217.885
	109.978	107.571	215.606	221.826
	108.079	104.124	169.957	238.987
Mean		**88.118**		**176.961**
SD		**39.618**		**41.225**
SEM		**3.235**		**3.366**

Figure 3. Day 11 7h in subjective night

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
72.084	68.823	137.811	157.066
66.972	102.558	118.633	168.101
72.339	63.805	111.527	136.11
86.53	54.801	177.157	166.542
65.236	48.475	172.171	164.146
45.175	77.6	148.558	158.744
59.984	72.807	152.292	144.297
67.098	71.483	128.063	114.862
74.975	84.709	150.718	131.118
47.788	94.105	185.559	242.551
45.283	114.322	171.367	218.476
72.401	88.296	127.054	246.608
62.597	125.136	165.024	234.695

67.013	124.444	134.998	247.069
68.427	108.711	119.831	227.011
67.698	121.182	202.957	234.053
54.604	93.766	170.094	226.511
73.013	82.151	207.042	241.518
125.649	128.668	210.275	224.648
73.533	122.574	216.299	219.397
74.37	107.319	199.692	236.063
52.305	115.113	226.515	159.163
77.227	138.42	221.587	169.53
73.764	142.733	238.566	156.295
87.591	142.999	197.299	199.129
102.608	136.87	206.248	196.916
83.127	86.174	191.614	205.56
94.552	89.427	145.379	155.355
80.344	132.576	201.773	224.05
58.954	166.958	201.991	191.777
75.33	136.006	176.519	156.509
129.571	164.481	181.108	141.062
93.569	130.517	157.126	150.118
68.839	127.399	151.238	156.702
63.074	148.796	120.06	118.893
92.426	111.982	121.019	180.086
121.566	85.427	117.004	179.024
82.542	73.334	96.025	182.419
86.055	47.809	90.453	172.657
98.968	81.037	194.378	187.225
89.578	49.945	105.568	157.574
66.038	41.36	134.871	206.221
82.975	25.996	139.004	98.964
44.933	17.037	138.64	99.32
74.218	144.854	98.613	125.036
42.71	62.559	185.357	103.534
63.687	113.847	180.431	142.77
106.071	20.594	228.434	129.136
71.097	180.752	237.445	89.505
88.071	86.455	174.926	64.012
130.514	151.987	220.056	77.204
80.021	146.336	131.352	68.193
154.952	173.854	144.111	105.766

	150.755	43.192	144.758	138.341
	88.616	63.056	128.373	162.426
	42.039	43.747	154.135	145.667
	36.032	72.657	115.487	114.565
	57.305	69.459	129.401	234
	63.808	116.329	152.349	219.072
	62.624	111.235	141.974	191.68
	43.914	118.26	126.745	230.199
	72.807	157.056	137.249	243.249
	77.073	150.801	118.823	233.342
	78.975	153.772	239.705	250.847
	58.793	122.898	252.842	251.541
	47.871	127.589	247.581	246.834
	41.883	133.501	239.823	234.211
	57.823	124.296	240.242	234.102
	56.808	119.362	239.017	243.757
	72.046	97.912	233.307	158.93
	55.533	87.685	212.861	166.609
	36.059	75.806	233.051	141.781
	70.051	58.534	230.844	207.825
	64.619	43.165	216.318	215.66
	50.5	50.858	201.957	212.49
Mean		**87.444**		**175.101**
SD		**35.218**		**47.501**
SEM		**2.876**		**3.879**

Figure 3. Day 11 1h in subjective day

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
58.112	8.574	111.562	82.912
74.848	17.234	79.841	111.501
80.553	2.885	115.202	124.037
48.775	17.011	121.096	92.745
59.586	36.369	80.185	77.686
28.693	65.832	121.438	109.292
77.357	28.254	106.447	102.46
55.99	14.892	162.607	36.386
79.189	18.266	143.78	32.86
88.571	14.489	170.552	26.118
46.115	10.176	131.591	39.465

54.945	11.858	135.978	41.216
98.419	12.004	138.973	81.176
62.434	20.986	126.133	18.695
69.214	20.763	155.188	21.262
61.437	58.19	145.316	23.685
78.451	54.585	112.429	15.085
40.157	44.278	82.429	14.499
57.957	55.454	91.911	15.72
72.887	76.448	93.661	85.568
52.6	67.195	124.618	76.896
55.663	88.397	114.253	57.375
28.492	72.825	94.644	96.567
59.573	42.872	93.486	114.849
39.257	99.407	92.009	100.39
41.062	85.958	117.471	57.99
46.091	106.098	76.172	50.307
80.535	110.37	104.976	52.342
85.579	76.485	90.431	84.515
70.909	102.841	103.14	68.416
51.792	72.549	84	65.066
57.736	51.779	144.591	116.771
47.042	49.655	138.912	110.566
44.6	39.816	134.306	108.789
55.015	64.934	134.827	99.375
73.173	36.972	139.956	87.648
46.836	44.981	97.946	107.372
60.256	69.306	149.591	127.16
56.681	38.487	112.984	94.796
43.666	12.327	104.27	101.506
98.726	14.596	97.718	87.883
89.535	13.742	126.214	90.003
64.33	17.107	98.624	98.314
75.907	11.324	148.483	119.851
86.853	17.841	160.5	121.469
94.998	17.868	132.82	114.577
60.187	16.139	137.069	116.426
51.049	17.138	125.789	103.646
36.671	18.312	122.617	86.83
32.641	13.905	125.442	71.319
55.226	24.274	114.717	82.821

	77.495	18.869	108.144	42.813
	106.049	42.041	127.635	140.072
	37.783	26.461	76.979	114.795
	45.471	17.351	113.198	99.336
	33.983	44.73	99.73	121.988
	68.327	28.6	116.573	104.831
	107.574	12.551	105.435	123.966
	65.035	113.206	97.324	101.892
	67.8	176.407	93.735	94.479
	54.112	96.081	82.417	94.368
	14.852	74.526	121.236	17.648
	15.734	82.226	97.947	11.573
	24.681	53.087	121.525	19.851
	41.635	67.914	110.834	39.696
	19.374	38.765	110.696	38.027
	19.002	91.269	101.08	40.527
	22.132	71.765	131.716	18.826
	18.648	56.798	122.33	19.845
	12.583	38.012	115.868	24.38
	21.469	75.703	117.152	21.527
	10.914	91.069	74.567	41.688
	16.402	80.818	122.683	30.439
	12.027	60.599	101.49	14.903
	14.795	79.237	118.147	38.215
Mean		**51.376**		**93.981**
SD		**29.554**		**37.544**
SEM		**2.413**		**3.066**

Figure 3. Day 11 7h in subjective day

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
50.024	17.527	89.973	72.529
51.684	27.141	92.957	79.03
52.656	37.901	68.289	76.942
41.114	20.015	106.235	90.603
56.792	16.292	81.89	89.916
47.511	19.223	105.078	96.973
40.876	21.321	102.265	65.227
34.917	14.874	130.841	70.245
30.549	51.095	153.807	68.936

33.161	37.95	89.112	73.77
38.673	28.416	141.144	78.637
47.003	21.354	87.897	76.697
52.723	21.573	113.779	48.833
59.909	10.882	178.875	66.506
38.535	13.002	108.358	60.818
42.008	25.216	138.244	139.528
41.333	62.494	96.496	92.975
36.533	129.167	129.561	151.961
35.178	84.618	132.543	89.624
58.629	95.606	140.737	86.602
43.86	111.041	93.343	78.458
41.074	69.909	164.521	42.011
37.315	79.981	157.985	37.137
44.567	57.926	123.461	34.727
43.745	85.579	210.056	46.321
44.438	91.061	137.851	49.308
59.738	128.481	187.353	38.822
42.813	64.022	127.634	37.599
56.661	82.56	78.02	36.404
53.182	37.885	65.186	37.011
77.64	58.937	108.159	51.332
54.652	44.268	78.96	29.699
63.503	56.101	101.969	54.092
75.234	56.557	120.254	41.31
45.506	64.616	150.601	41.798
44.844	60.976	111.075	37.527
45.825	104.798	109.416	118.058
58.802	50.056	111.09	106.975
37.293	43.427	124.926	117.018
29.392	41.712	97.017	46.257
43.089	67.38	99.717	57.752
37.697	56.839	67.856	38.743
37.267	54.415	102.404	62.654
43.926	54.797	130.831	59.36
37.904	63.137	104.067	50.462
35.098	45.791	83.004	52.455
37.597	47.405	97.414	54.352
40.173	43.575	79.684	54.796
43.07	50.192	92.435	61.273

	44.92	33.172	117.089	53.451
	38.788	36.551	94.667	59.526
	48.265	38.356	76.081	134.603
	45.519	48.62	85.447	124.079
	61.457	51.712	87.655	118.481
	104.783	49.57	102.212	113.718
	86.672	52.209	104.627	138.649
	79.818	46.079	101.52	129.579
	110.962	37.798	90.105	149.035
	75.061	39.904	88.527	134.17
	72.415	49.623	85.383	170.803
	87.328	33.895	109.069	105.427
	87.571	50.327	88.31	116.017
	84.149	41.993	99.737	104.177
	68.676	51.606	154.511	99.945
	78.401	33.102	91.99	125.895
	58.21	50.723	116.252	104.176
	52.444	53.837	76.974	79.572
	56.415	47.836	72.553	80.862
	55.251	49.421	70.834	75.413
	55.051	48.307	125.616	69.691
	48.266	82.273	121.83	48.376
	36.334	80.305	132.758	44.248
	55.827	70.098	138.395	73.044
	21.669	66.664	122.768	95.758
	13.494	72.084	125.593	69.813
Mean		**52.124**		**94.356**
SD		**21.701**		**35.279**
SEM		**1.772**		**2.881**

Figure 3. Day 12 1h in subjective night

Cytoplasm Pixel Intensity		Nucleus Pixel Intensity	
109.711	65.003	242.941	145.084
49.162	72.106	243.914	169.515
52.359	57.241	225.941	163.38
58.949	68.567	219.62	153.55
36.568	82.409	218.365	157.417
54.003	65.741	196.977	102.955
91.015	117.35	198.714	89.546

92.14	135.635	208.071	96.697
74.965	77.894	194.838	82.478
86.851	55.172	200.199	119.221
76.896	147.987	196.736	116.487
63.337	157.476	211.242	101.333
36.534	100.7	235.184	167.019
57.191	90.671	231.886	142.4
62.141	60.523	225.013	120.136
59.14	76.032	222.519	230.402
57.149	129.101	245.909	245.368
66.803	154.142	237.133	231.73
82.401	192.359	233.969	219.679
105.108	140.744	198.324	232.622
53.623	105.658	208.604	232.67
48.696	195.745	236.081	222.836
56.911	148.83	224.553	238.273
33.152	202.791	237.016	242.618
65.654	229.909	229.61	240.438
52.009	243.23	237.719	232.84
63.727	154.532	216.062	235.543
58.573	136.046	234.925	235.843
69.337	188.019	236.717	239.937
91.747	185.657	206.769	238.579
48.471	174.672	222.951	243.251
46.528	108.345	214.521	236.302
50.582	167.861	232.181	241.971
57.938	127.352	246.117	220.123
77.237	163.89	224.622	201.715
48.193	206.648	241.138	217.755
79.716	155.969	241.52	235.205
38.807	142.667	220.301	239.374
43.73	123.695	228.23	239.996
59.456	129.315	240.59	209.575
69.848	168.959	241.213	203.474
63.302	115.196	237.406	205.807
64.361	92.8	252.877	242.637
54.489	145.522	254.364	227.847
57.986	142.172	251.12	233.038
45.789	109.11	250.078	224.834
55.351	122.55	251.039	184.878

	71.145	146.734	254.721	236.332
	22.517	151.876	253.809	219.915
	49.421	129.203	254.369	206.613
	58.821	136.709	250.321	227.572
	65.154	82.367	252.848	216.75
	84.3	128.072	253.682	225.501
	71.024	141.654	252.33	218.225
	104.575	129.139	253.238	157.027
	64.124	149.941	254.783	175.422
	47.305	142.177	255	161.577
	98.157	152.133	254.618	166.973
	93.162	119.764	251.423	159.891
	125.086	134.647	254.43	168.785
	156.228	130.943	202.219	154.493
	92.995	127.567	207.714	165.947
	98.394	110.069	223.079	171.627
	101.432	156.656	163.281	131.793
	72.462	131.005	155.986	130.814
	40.069	126.66	160.537	154.567
	79.672	160.309	171.251	108.225
	94.3	94.718	161.432	94.152
	46.321	120.202	146.392	83.73
	41.243	111.547	118.333	128.686
	52.814	121.722	148.886	125.041
	53.252	124.516	142.862	142.512
	44.804	147.671	163.543	95.002
	34.463	161.42	190.729	95.347
	42.936	163.196	176.489	84.263
Mean		**99.336**		**201.302**
SD		**46.547**		**48.025**
SEM		**3.801**		**3.921**

Figure 3. Day 12 7h in subjective night

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
90.017	163.426	195.835	128.368
94.414	161.744	183.601	113.135
58.014	107.177	181.41	106.184
92.204	188.377	202.173	241.243
75.227	139.306	195.434	237.642

96.473	107.902	196.846	212.169
136.498	107.769	167.728	233.077
105.62	177.658	151.557	224.645
87.732	138.254	140.902	247.796
99.436	138.063	222.632	216.668
101.91	129.686	222.357	201.916
71.53	113.152	223.11	226.935
106.109	129.542	154.616	237.73
89.65	120.389	188.816	247.788
84.642	134.585	191.097	245.75
103.788	120.062	177.242	228.758
82.886	96.801	192.145	241.036
94.453	126.392	157.618	210.571
68.619	100.662	187.593	197.599
100.009	94.463	155.768	193.834
66.706	117.201	142.814	191.373
102.62	89.615	188.675	250.746
132.112	96.812	171.464	254.533
83.129	76.402	197.63	227.38
90.378	114.772	186.443	117.97
128.778	76.258	188.088	125.003
78.78	105.3	180.955	131.988
108.54	88.418	166.662	253.2
129.851	115.922	171.195	252.633
156.371	112.549	135.953	253.499
85.928	65.377	231.339	241.115
87.276	82.699	198.793	229.348
81.991	88.413	203.506	240.405
94.432	54.481	218.377	223.243
94.422	80.127	236.219	179.533
59.304	56.003	179.124	196.554
97.95	109.081	208.101	192.097
72.83	97.726	153.26	207.32
67.955	101.518	211.447	194.903
97.163	103.736	222.607	227.242
67.867	142.539	194.227	233.412
63.798	77.16	177.42	225.066
114.811	96.964	233.83	232.2
135.135	100.049	206.116	232.933
93.099	117.303	223.532	188.215

	174.315	98.52	229.61	241.418
	195.158	64.752	194.975	221.442
	134.501	108.234	185.333	220.756
	166.822	97.202	246.109	127.805
	177.468	69.635	228.921	97.075
	160.837	129.423	193.898	104.824
	186.234	189.659	186.107	111.691
	156.125	152.174	225.11	115.571
	107.544	127.999	182.757	104.176
	193.912	136.556	173.939	111.055
	175.276	139.619	211.132	105.733
	198.878	96.853	164.421	96.129
	155.702	83.156	223.776	103.667
	142.722	75.484	231.61	111.005
	152.992	100.028	181.428	102.927
	132.965	80.333	174.794	97.952
	125.529	105.485	223.625	107.473
	85.825	100.122	156.79	108.967
	160.035	83.622	109.857	115.735
	110.41	84.05	153.905	143.795
	162.299	93.279	156.036	119.063
	163.212	72.314	90.734	196.242
	143.29	107.585	129.601	183.393
	148.1	81.891	120.748	191.875
	146.084	102.4	155.855	77.635
	147.264	102.117	111.473	111.938
	142.699	137.236	110.115	76.059
	102.762	111.94	133.042	217.889
	147.092	130.702	143.21	160.212
	144.374	110.342	167.191	183.559
Mean		**112.703**		**181.828**
SD		**33.208**		**47.086**
SEM		**2.712**		**3.845**

Figure 3. Day 12 1h in subjective day

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
17.563	59.274	193.49	38.844
19.599	57.761	170.717	38.453
18.521	93.427	206.531	47.333

14.026	56.612	169.741	88.76
19.561	50.699	193.15	64.262
20.905	55.544	192.867	70.663
16.368	83.158	138.529	107.913
25.365	61.189	142.588	107.678
21.787	79.731	125.839	83.955
17.225	67.778	182.432	101.225
24.591	76.199	160.723	115.304
17.68	93.436	138.368	99.691
17.406	69.435	76.585	70.738
24.826	70.834	85.378	87.605
20.401	62.781	86.906	86.447
16.276	51.106	175.191	111.082
25.855	46.75	170.378	174.418
24.324	33.214	120.687	115.488
23.039	55.797	149.827	75.849
22.45	79.149	83.133	89.064
17.368	91.824	133.028	81.985
23.796	97.886	68.325	57.138
27.092	110.566	91.936	65.179
21.539	63.778	74.429	52.275
25.908	95.132	177.469	74.304
30.776	70.246	129.118	46.093
21.305	85.851	194.173	51.75
16.792	97.877	52.039	101.657
21.798	79.304	57.473	87.182
14.685	86.685	61.244	103.2
20.876	38.769	191.009	71.951
21.232	59.52	131.467	92.405
26.878	47.966	168.778	80.499
15.374	63.494	114.165	75.064
58.712	50.99	119.613	86.074
69.944	52.989	158.989	60.895
48.824	59.098	114.129	62.658
47.048	49.684	133.8	95.926
55.379	43.822	121.713	91.073
57.409	47.469	101.738	141.328
55.819	55.488	140.374	116.675
52.742	63.684	114.931	155.966
65.421	47.56	156.05	124.933

	40.762	57.929	139.615	169.398
	42.898	76.03	136.175	120.868
	41.599	56.345	137.723	141.455
	47.912	60.147	100.793	117.7
	44.356	87.93	130.07	152.75
	73.652	54.346	148.514	169.635
	38.649	74.396	92.157	69.714
	40.623	117.708	163.453	103.6
	53.661	97.056	146.504	138.493
	40.974	54.474	135.09	135
	50.03	92.167	137.653	72.871
	28.386	73.445	156.683	169.707
	31.38	127.339	141.515	143.534
	39.652	76.326	189.425	145.935
	35.407	73.775	158.272	186.6
	33.792	70.934	119.494	172.018
	39.684	66.96	157.116	134.625
	32.325	20.29	95.258	80.377
	46.553	23.079	82.14	124.796
	51.952	22.188	133.566	99.049
	53.394	21.44	134.299	86.899
	45.104	14.14	129.942	82.439
	41.849	17.074	80.29	66.903
	65.86	15.172	98.522	101.672
	65.845	18.655	107.145	102.58
	97.337	22.026	65.914	72.351
	101.084	17.535	31.713	211.402
	98.557	17.243	32.793	156.771
	62.816	47.59	24.978	90.59
	43.472	47.723	24.416	58.851
	75.251	45.062	31.101	50.456
	61.555	43.604	24.766	67.789
Mean		**49.764**		**112.026**
SD		**25.738**		**43.929**
SEM		**2.102**		**3.587**

Figure 3. Day 12 7h in subjective day

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
73.879	20.915	131.866	47.094

73.442	18.858	122.708	38.091
67.176	15.412	93.966	35.892
85.262	18.691	110.993	44.283
66.525	19.561	125.6	46.064
94.108	19.517	118.538	48.532
81.004	22.333	132.305	99.678
80.526	21.414	115.628	133.768
76.019	18.742	195.731	87.988
65.831	26.412	133.351	80.502
64.426	70.366	97.696	106.712
87.052	75.583	117.963	82.527
66.869	78.003	143.42	90.125
68.915	65.154	155.977	111.878
85.711	70.045	175.357	71.688
66.745	64.318	158.25	108.06
58.03	66.301	109.612	94.66
54.108	53.391	122.129	88.763
94.722	33.5	109.012	86.132
83.04	60.812	115.988	56.432
71.658	57.875	124.412	82.017
92.419	38.095	131.74	65.55
54.555	42.893	160.556	90.17
91.128	27.098	131.014	84.775
80.699	61.833	92.493	120.648
79.096	51.191	129.434	126.801
76.016	90.086	103.122	116.568
73.364	63.211	170.397	78.042
59.591	46.395	154.25	81.529
67.281	47.5	109.081	71.726
84.876	44.521	138.238	60.804
87.633	60.174	142.116	66.071
75.369	42.313	86.129	55.439
72.538	62.103	193.745	64.878
90.228	63.544	185.916	51.054
93.765	46.167	152.729	73.687
92.258	36.923	159.683	144.677
78.253	47.94	114.568	144.628
59.728	50.444	132.25	119.1
94.76	43.511	139.985	153.898
78.726	42.558	142.47	132.896

	78.456	46	138.867	132.373
	54.314	50.788	150.3	121.414
	64.006	63.435	132.532	109.84
	47.714	38.6	111.35	167.102
	46.681	40.191	109.546	121.012
	42.814	64.756	115.118	119.37
	42.425	46.986	108.676	155.557
	46.875	69.831	143.421	117.979
	55.768	88.933	117.122	166.262
	19.637	50.119	145.759	158.574
	19.504	115.15	101.973	131.265
	19.19	62.864	70.069	126.976
	22.199	49.515	76.699	102.713
	18.888	107.693	103.644	130.551
	18.161	52.7	85.013	96.068
	27.758	100.937	103.128	131.385
	31.48	91.68	108.998	95.638
	76.258	85.087	124.788	140.002
	73.799	72.858	93.118	110.685
	82.237	87.031	101.607	88.351
	49.468	72.306	98.544	161.192
	14.384	52.338	94.799	135.284
	21.141	70.739	110.76	131.915
	26.888	113	87.998	84.648
	33.534	65.547	106.537	78.032
	24.004	77.671	47.437	100.006
	36.393	80.107	32.434	106.379
	20.533	84.713	31.996	94.075
	26.852	74.824	32.109	143.824
	28.376	89.967	20.728	92.688
	30.533	98.494	26.964	67.001
	18.713	72	36.452	137.155
	15.265	66.926	39.944	81.333
	18.116	74.786	45.625	90.872
Mean		**58.56**		**107.372**
SD		**24.652**		**36.724**
SEM		**2.013**		**2.999**

Figure 3. Day 13 1h in subjective night

Cytoplasm Pixel Intensity		Nucleus Pixel Intensity	
64.827	105.454	213.672	118.792
59.697	103.129	174.274	102.648
54.591	81.433	216.118	112.928
51.209	139.753	167.741	116.058
59.352	136.342	148.67	101.207
42.238	146.962	156.243	123.374
68.168	131.38	215.653	209.269
61.857	114.16	177.909	150.891
100.322	124.582	150.736	205.539
84.424	29.871	182.566	199.827
59.579	60.852	150.752	222.91
37.14	67.293	162.872	181.246
69.759	45.547	146.015	220.099
61.199	55.695	124.267	206.143
39.912	81.632	119.282	182.857
90.423	93.231	136.253	193.385
85.264	76.432	222.329	209.88
70.438	48.726	124.004	191.678
30.861	43.936	125.023	166.647
67.181	39.873	135.28	146.643
99.18	37.512	62.52	140.672
60.926	53.471	165.922	200.809
76.968	61.399	144.875	223.908
70.73	71.585	162.833	213.653
77.19	58.457	147.429	125.416
47.824	50.457	147.778	77.364
42.125	44.961	164.444	118.438
46.053	35.791	211.814	109.516
53.551	35.486	190.616	105.668
56.158	52.919	184.165	97.767
72.944	34.797	188.096	87.667
57.975	60.698	187.534	126.181
67.872	43.241	168.58	95.018
73.85	77.222	199.048	110.277
71.298	82.87	186.792	107.11
87.529	38.613	177.986	86.645
92.706	53.831	182.649	120.792
70.256	77.297	136.589	133.194

	63.684	52.938	182.071	120.213
	64.938	47.142	161.465	124.316
	17.417	69.556	165.288	94.114
	17.221	42.449	180.558	114.257
	42.102	78.356	174.426	134.034
	79.395	79.217	132.66	112.5
	87.629	73.512	161.714	111.747
	51.498	83.917	122.438	120.081
	74.697	82.292	147.926	93.735
	44.623	138.084	134.742	101.486
	118.71	145.707	125.776	163.962
	63.791	84.596	113.04	103.48
	146.184	133.198	98.945	95.701
	181.097	86.178	243.098	102.954
	74.241	76.611	244.28	137.07
	80.745	111.06	245.872	114.758
	193.035	123.563	250.834	103.597
	193.555	68.448	254.305	135.183
	136.556	40.567	251.04	107.759
	135.15	50.559	247.371	120.375
	156.887	37.846	253.321	128.753
	149.75	74.638	249.755	106.7
	113.714	120.359	250.482	160.655
	183.366	96.198	246.129	227.493
	65.624	114.672	249.246	193.782
	157.009	84.791	234.444	191.817
	79.645	59.936	252.571	189.046
	150.023	83.733	226.024	147.914
	180.061	79.824	200.698	130.486
	117.216	79.487	170.762	103.681
	99.318	83.83	150.235	142.587
	104.12	97.412	190.314	189.805
	156.99	80.108	194.286	179.386
	151.178	104.056	182.917	182.436
	121.727	93.13	110.316	192.126
	141.114	91.958	120.101	236.768
	125.334	81.206	143.744	192.74
Mean		**82.873**		**161.148**
SD		**37.699**		**47.201**
SEM		**3.078**		**3.854**

Figure 3. Day 13 7h in subjective night

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
84.149	57.622	172.528	213.984
104.075	43.377	162.214	228.779
103.928	37.178	171.713	198.629
108.621	43.784	132.677	221.317
99.946	41.891	171.63	208.381
98.171	37.663	180.685	222.751
81.119	38.84	198.692	248.032
111.258	46.476	168.356	207.808
114.204	76.091	175.078	236.496
41.401	88.686	200.35	176.386
67.025	127.919	162.095	210.571
77.43	47.926	217.025	225.367
92.531	76.038	190.738	207.379
94.944	101.582	177.147	201.683
73.595	32.453	144.754	203.678
66.93	30.666	69.061	219.389
71.654	45.468	69.849	243.214
46.314	47.046	72.407	233.818
92.68	53.483	130.147	221.725
57.011	85.745	126.615	198.88
108.422	31.177	123.518	213.41
40.148	74.664	151.069	168.024
43.891	71.182	105.636	191.5
57.379	40.67	86.326	193.117
104.871	51.807	172.561	227.061
78.714	47.794	155.09	182.392
116.79	84.494	184.965	229.724
152.7	46.796	157.392	226.537
179.812	106.324	158.532	219.974
166.238	105.995	203.571	168.408
156.943	64.593	130.415	235.811
96.194	64.913	171.627	217.015
125.923	67.967	188.5	208.396
139.894	65.399	169.064	140.5

113.426	59.072	148.611	115.22
76.118	58.904	125.713	146.502
70.161	57.023	179.509	159.018
116.276	109.591	155.871	144.992
152.472	36.941	172.344	131.755
117.366	45.685	121.572	194.52
119.405	73.855	143.707	173.892
142.47	46.353	157.135	165.754
64.123	47.781	133.641	141.453
108.392	43.782	148.278	119.234
83.047	105.94	141.398	105.962
133.07	105.94	168.972	138.465
126.624	54.253	174.233	129.489
108.439	56.35	185.122	117.893
86.615	49.506	178.196	149.864
51.229	61.984	162.73	138.067
102.658	72.203	140.362	135.401
102.755	36.238	211.966	179.181
72.157	62.315	251.105	137.494
66.196	75.422	234.385	111.87
66.202	94.109	245.574	197.547
54.494	74.547	219.004	251.633
80.852	67.123	191.567	165.133
84.412	118.304	236.51	167.9
63.879	67.692	212.272	143.131
55.998	70.045	236.847	146.615
78.671	90.163	245.517	160.323
56.223	75.258	246.103	115.421
90.11	88.9	183.25	171.473
55.487	81.106	226.246	122.583
63.274	117.243	180.686	156.735
89.68	44.776	199.343	145.819
35.766	76.899	174.953	167.929
42.034	96.77	213.244	170.992
50.572	120.581	187.309	163.598
40	101.65	217.14	125.2
74.278	76.926	210.834	87.428
57.75	51.526	213.614	130.967
20.023	118.83	226.509	119.064
29.26	64.7	235.206	168.028

	51.974	115.341	201.172	130.522
Mean		**77.761**		**175.227**
SD		**31.447**		**41.444**
SEM		**2.568**		**3.384**

Figure 3. Day 13 1h in subjective day

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
14.577	54.449	45.015	211.423
26.115	75.912	58.706	153.256
18	61.035	52.789	188.872
16.62	58.915	44.466	173.238
12.161	72.656	37.41	158.749
16.281	30.582	42.488	160.188
21.141	53.519	30.6	162.891
20.284	98.848	30.247	235.546
10.167	75.365	24.763	125.765
13.781	62.433	22.08	146.793
16.177	68.426	21.784	108.145
11.924	56.287	17.587	151.246
10.069	49.889	27.086	195.977
22.593	69.308	25.034	144.499
16.303	60.403	21.866	148.636
19.769	46.633	99.271	129.404
11.137	51.814	128.666	150.059
16.573	54.865	140.865	151.263
21.689	51.899	156.665	98.44
18.84	40.76	115.959	167.592
19.885	45.964	97.979	99.581
16.436	40.578	86.845	116.769
22.324	46.867	87.6	106.041
17.134	37.541	63.299	110.423
21.495	35.283	93.191	175.067
17.606	21.762	164.315	143.265
14.533	25.947	82.755	175.438
14.41	17.699	89.3	106.879
25.527	15.913	139.492	130.356
54.281	12.105	124.366	110.685
19.647	15.949	113.627	166.99
19.874	18.034	99.704	90.754

16.626	15.557	91.662	123.025
14.813	15.996	96.808	160.931
12.63	49.895	96.901	150.758
13.57	34.244	100.307	138.305
18.517	28.822	134.456	108.894
15.558	42.961	165.338	75.624
29.097	46.668	115.399	123.72
33.57	44.167	128.912	136.943
38.939	32.012	129.305	138.116
38.765	51.378	120.214	130.345
28.664	32.917	130.205	130.755
31.741	31.804	75.605	136.882
36.765	58.719	119.344	146.025
28.626	54.85	109.585	130.324
69.004	38.631	127.354	95.709
56.13	37.204	125.72	168.62
42.265	62.577	172.432	115.081
18.407	53.755	119.642	129.075
80.771	52.667	172.451	113.987
34.098	64.491	167.303	168.282
53.304	68.303	172.408	127.315
73.32	38.449	159.864	92.43
55.807	34.613	160.315	101.39
52.873	32.446	76.891	152.27
42.748	40.788	138.909	129.431
44.538	70.913	189.263	120.166
45.876	65.361	172.855	110.17
43.482	48.885	188.854	106.513
40.913	60.122	178.733	155.649
37.807	49.885	146.045	145.381
38.928	47.899	196.037	124.113
45.047	77.247	90.398	129.649
58.67	48.513	72.097	146.716
41.771	46.307	78.565	114.649
27.006	36.129	93.738	83.485
35.537	55.087	105.606	112.914
34.214	42.616	124.364	91.5
67.704	51.015	83.489	116.396
46.111	35.152	45.186	141.64
34.227	43.617	65.053	99.265

	63.754	39.621	132.053	158.443
	114.712	44.281	131.924	113.7
	106.183	43.981	111.116	133.736
Mean		**39.724**		**119.474**
SD		**20.385**		**42.745**
SEM		**1.664**		**3.49**

Figure 3. Day 13 7h in subjective day

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
24.709	18.896	83.832	31.612
21.331	7.08	95.434	27.585
26.895	12.566	79.038	29.346
26.971	11.299	62.558	44.104
24.354	3.121	63.702	56.734
25.685	42.064	54.645	39.787
54.232	14.721	45.353	30.109
30.958	22.413	105.149	33.28
41.136	14.461	65.987	34.567
42.769	13.395	70.607	19.078
38.457	11.061	45.465	25.778
35.679	11.207	64.337	24.034
40.63	13.101	115.012	26.168
41.915	13.963	61.344	34.202
69.512	24.054	54.502	22.378
56.544	22.256	114.633	34.257
56.544	27.886	134.949	27.531
53.199	15.307	80.02	26.325
53.294	15.819	93.48	28.638
35.789	13.345	117.695	21.178
26.813	12.498	43.465	25.871
40.939	16.703	127.603	35.576
29.922	25.22	103.713	42.287
36.745	22.863	61.679	32.088
56.425	13.507	95.095	61.682
28.898	19.435	53.568	30.889
24.679	17.363	80.962	77.992
23.385	21.448	70.541	82.833
28.859	17.761	85.291	70.572
29.718	21.608	59.506	60.983

28.136	56.25	44.727	66.288
27.986	40.205	39.297	75.156
14.262	24.147	39.87	71.994
27.043	56.777	23.969	89.218
27.032	76.454	31.32	86.062
27.242	30.874	33.566	101.893
21.98	29.36	14.978	75.7
15.505	28.105	13.747	71.227
32.586	27.747	19.963	94.434
38.913	11.477	39.329	99.382
31.435	41.23	37.644	102.089
42.221	49.564	28.591	93.324
40.188	44.885	47.857	82.295
26.776	30.631	52.11	92.488
22.64	55.345	23.742	83.218
27.441	38.93	30.166	90.914
32.491	56.913	34.447	81.394
32.959	20.893	31.196	66.477
25.909	19.298	40.897	67.161
21.456	40.275	38.702	64.328
15.625	33.341	31.802	69.086
37.542	63.365	46.557	83.256
42.9	24.337	30.355	74.328
42.841	27.696	33.057	77.218
30.225	21.786	45.183	84.877
47.194	40.449	37.255	85.144
37.113	26.432	22.931	63.109
36.438	55.926	36.046	203.582
49.614	36.454	27.33	103.381
29.574	28.679	36.635	114.249
35.12	35.885	19.837	63.912
32.268	44.948	38.455	77.228
41.08	45.203	31	61.699
20.144	56.021	27.222	67.071
11.478	50.783	28.435	73.676
4.696	34.858	18.515	64.143
11.268	10.634	33.015	58.813
11.864	14.601	47.998	54.041
17.828	35.215	22.164	71.037
14.856	30.626	27.314	63.791

	16.885	19.546	29.357	68.336
	12.751	18.969	20.843	68.399
	9.692	16.354	24.533	62.844
	17.029	33.421	25.684	57.116
	9.502	12.604	23.256	74.842
Mean		**29.604**		**57.052**
SD		**14.339**		**29.905**
SEM		**1.171**		**2.442**

Figure 4. Day 11 first time-point in LL

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
56.689	27.899	131.081	43.579
54.225	41.426	156.264	59.707
56.953	37.717	123.052	66.211
50.471	57.888	87.117	58.579
90.088	57.126	80.55	68.315
102.648	45.721	82.459	52.002
63.314	26.169	110.662	52.98
84.263	38.576	87.398	39.501
71.33	55.404	99.184	30.78
82.21	30.715	28.593	96.976
53.572	46.46	35.595	67.044
39.749	42.362	31.864	103.459
62.302	44.513	41.374	87.901
48.839	39.586	48.717	66.793
98.075	61.872	38.127	84.529
76.934	41.32	28.924	87.329
38.906	36.746	34.95	92.593
42.924	40.204	34.815	93.587
34.805	48.685	30.657	58.125
47.731	37.602	28.576	70.564
60.806	40.575	33.541	53.49
60.635	60.165	33.754	57.46
53.765	54.236	32.384	71.154
78.946	40.418	38.714	62.408
64.824	43.086	46.418	75.682
38.499	42.264	36.699	63.044
71.09	47.127	31.078	76.103
58.078	42.809	32.231	89.528

31.794	9.698	24.226	70.991
25.445	7.732	30.878	68.861
26.171	10.909	26.927	75.96
31.574	10.573	28.313	84.41
32.894	7.214	26.997	79.295
41.622	2	36.644	76.385
46.342	15.678	33.446	58.837
42.545	9.364	30.8	70.722
48.682	45.129	154.861	63.937
70.701	21.524	145.548	79.039
59.999	30.49	122.866	45.496
49.899	33.646	125.577	80.667
58.16	58.267	127.175	83.04
54.761	49.627	101.152	75.789
236.87	59.863	56.931	71.942
79.123	65.036	60.748	76.119
67.89	42.794	65.19	77.974
67.014	38.397	101.719	98.687
106.353	42.459	69.59	84.968
37.746	52.311	101.5	83.622
52.42	57.489	104.408	57.639
93.048	9.536	114.386	72.918
118.861	22.039	107.619	71.38
90.766	28.637	85.063	57.436
49.752	37.794	72.918	54.083
49.437	58.429	59.083	86.266
50.447	54.169	125.47	72.947
40.705	49.922	145.361	78.782
73.594	43.362	106.612	81.533
58.632	35.161	84.459	84.57
51.385	33.735	144.593	91.725
47.402	85.665	122.273	81.957
70.183	77.37	106.568	66.593
61.586	76.302	102.592	85.853
57.087	48.795	83.383	90.003
45.296	48.529	97.412	81.34
39.769	44.242	80.572	78.64
63.546	63.797	89.794	85.616
81.333	40.368	126.824	90.851
76.248	38.728	99.242	78.254

	30.616	48.772	130.649	78.243
	52.225	26.572	137.55	117.326
	39.175	48.373	100.372	87.743
	37.563	52.6	116.414	111.915
	20.129	70.683	102.089	96.315
	23.654	61.361	85.88	100.137
	39.153	32.213	83.057	99.309
Mean		**50.722**		**77.24**
SD		**25.458**		**30.423**
SEM		**2.079**		**2.484**

Figure 4. Day 11 second time-point in LL

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
56.923	16.059	29.473	97.969
50.886	6.638	20.796	104.755
43.067	11.611	24.509	115.932
55.456	11.95	35.991	79.41
41.105	17.874	35.517	81.758
44.092	15.514	50.086	92.01
24.124	13.779	27.723	80.99
20.598	8.586	27.393	81.632
23.268	13.127	33.649	81.986
26.846	28.746	35.234	114.691
55.184	27.193	29.802	101.171
47.362	40.006	32.366	85.311
71.356	27.272	37.006	62.575
30.922	47.577	34.58	68.98
31.548	88.238	38.491	56.842
51.92	41.141	31.438	86.596
55.621	37.612	33.924	78.294
56.537	36.851	31.974	128.161
59.125	69.796	38.34	95.807
72.545	58.975	35.856	84.032
43.241	61.256	36.792	86.155
64.681	50.537	35.929	101.799
36.621	28.631	35.413	125.243
82.279	31.239	41.478	118.94
86.159	42.422	37.254	158.144
95.739	48.144	31.688	99.676

88.631	48.317	43.167	178.623
87.582	53.105	39.651	145.429
67.583	54.981	39.996	139.875
67.111	78.089	35.954	118.371
42.102	62.489	37.041	145.748
34.158	66.817	27.529	124.61
50.372	39.312	38.52	128.88
127.507	37.717	35.624	98.248
81.763	76.902	26.955	116.131
57.952	35.26	33.994	126.254
31.06	46.314	32.989	98.167
74.24	50	12.302	94.787
56.151	53.289	8.813	99.166
54.514	40.598	47.973	70.915
83.839	80.726	70.037	76.929
41.005	41.059	35.403	82.678
63.84	109.448	78.844	67.538
82.272	72.648	122.156	57.275
37.496	74.473	77.735	98.806
90.233	77.742	76.463	35.142
41.808	88.984	69.055	49.661
27.992	79.281	58.404	53.244
66.972	62.222	83.001	125.526
105.455	110.569	109.885	96.75
52.187	65.947	99.818	118.326
87.37	57.125	143.076	120.596
45.331	37.733	108.859	108.279
49.684	37.119	102.236	89.178
73.855	49.547	114.251	155.38
50.951	59.176	97.972	131.361
87.824	52.669	116.148	130.959
77.93	72.457	96.047	115.096
65.548	32.485	101.998	103.925
100.898	52.658	107.311	111.908
56.709	85.174	108.001	83.13
72.338	64.046	86.011	115.246
112.366	75.029	90.847	91.119
88.386	78.523	122.876	123.708
83.49	72.626	104.386	116.405
78.273	69.398	85.016	128.272

	64.653	83.338	142.01	151.494
	19.837	71.161	93.548	82.202
	33.878	68.547	111.267	118.522
	19.918	75.281	96.33	109.604
	11.248	59.722	93.229	100.321
	17.953	54.977	86.744	107.108
	41.929	133.992	115.022	89.693
	18.125	70.36	114.318	126.401
	15.649	70.657	126.866	92.207
Mean		**55.907**		**83.376**
SD		**25.263**		**37.473**
SEM		**2.063**		**3.06**

Figure 4. Day 11 third time-point in LL

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
34.893	62.335	72.579	26.21
33.261	69.389	87.055	27.703
30.351	77.438	87.61	24.634
26.52	68.262	84.043	28.2
36.496	41.472	94.442	36.954
43.241	43.872	102.88	31.81
51.942	39.578	150.624	21.662
55.153	39.941	73.436	35.325
38.984	31.598	125.872	30.809
51.186	23.078	83.515	25.495
48.85	81.747	83.683	37.536
41.728	79.869	86.895	39.093
45.405	58.489	96.657	33.378
84.977	94.85	103.938	37.029
30.853	87.831	88.825	33.052
36.436	71.364	71.283	36.297
32.39	46.647	76.816	30.299
22.594	39.966	87.733	29.844
18.613	91.041	84.888	147.029
17.337	72.792	82.353	118.076
10.328	51.178	83.88	130.646
22.62	73.33	90	146.462
38.257	39.757	85.929	103.322
75.451	130.531	80.558	118.119

79.376	67.676	73.427	117.742
83.826	85.165	67.458	124.527
51.579	88.854	54.096	107.09
10.844	107.722	107.324	98.444
16.691	72.775	114.27	104.769
32.38	53.911	113.241	88.429
25.273	110.671	98.664	74.168
25.786	73.233	129.064	90.035
30.469	72	98.431	75.567
27.073	54.497	126.245	81.342
45.687	51.558	87.105	71.474
44.863	55.925	85.762	73.985
60.18	91.552	114.433	89.23
34.209	74.925	79.307	116.058
64.514	71.042	90.663	79.704
33.397	48.321	81.237	78.307
17.81	57.271	91.894	90.056
23.879	75.266	108.795	78.381
20.823	59.028	102.074	100.262
22.085	40.136	131.244	116.908
41.931	54.204	119.592	83.868
43.269	51.799	91.635	107.588
24.298	56.185	90.476	79.007
22.774	38.723	119.09	101.574
25.618	40.987	129.76	107.395
29.002	42.821	101.329	120.027
29.486	45.757	119.545	100.847
40.989	57.408	121.907	115.347
30.346	40.137	132.418	84.439
33.296	51.67	123.765	107.543
41.264	48.524	79.325	93.333
73.948	50.101	91.038	96.248
49.558	48.792	93.089	118.243
55.509	32.296	98.46	109.836
80.653	34.956	122.025	129.752
71.446	35.136	91.689	133.36
59.209	38.68	121.051	112.351
71.764	62.524	80.602	99.679
72.692	59.271	107.473	121.072
71.433	56.276	150.917	89.482

	63.386	28.504	128.049	98.999
	71.28	41.018	122.923	106.741
	47.7	59.983	22.138	112.148
	75.581	46.704	25.87	91.664
	77.42	52.586	35.826	106.267
	46.783	51.896	26.764	89.423
	75.426	47.667	22.719	96.065
	66.763	45.152	23.793	163.372
	67.714	62.046	41.583	86.697
	59.016	53.13	32.715	109.301
	72.275	21.542	27.319	79.796
Mean		**51.699**		**88.347**
SD		**21.516**		**32.872**
SEM		**1.757**		**2.684**

Figure 4. Day 11 fourth time-point in LL

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
57.167	38.345	127.535	49.573
59.95	24.53	177.67	35.845
33.867	15.29	173.542	29.051
24.74	31.59	97.661	35.824
53.849	39.055	76.493	36.923
45.606	39.391	61.054	22.173
61.532	53.938	81.808	28.253
71.994	32.03	91.43	28.283
59.13	50.009	88.568	29.923
50.357	11.713	99.311	38.597
84.39	22.948	88.836	41.576
49.166	29.057	113.6	29.933
83.252	20.552	137.491	27.221
47.334	25.255	143.289	33.533
57.328	35.083	134.323	44.484
61.454	32.871	80.076	27.593
93.802	13.562	81.82	22.228
69.84	73.099	85.995	34.993
78.534	59.972	65.335	83.238
127.823	38.541	88.039	94.974
45.504	23.181	62.807	133.515
49.825	27.967	76.98	118.979

83.642	22.287	66.537	84.278
47.731	24.327	89.604	98.388
64.278	29.201	79.298	55.212
48.516	26.027	58.127	118.872
97.229	25.676	73.675	82.44
43.132	41.046	63.707	137.195
56.984	59.804	62.392	81.014
70.108	46.916	81.977	91.129
33.206	83.041	46.284	116.067
62.427	49.649	34.541	101.216
53.114	48.67	44.762	124.479
58.233	55.178	64.457	62.901
66.415	48.198	71.714	62.847
132.376	44.703	63.386	67.926
60.266	49.67	64.393	71.071
33.402	56.523	69.585	92.087
59.161	66.541	77.259	86.363
68.153	47.315	139.178	100.453
100.632	21.936	181.869	75.428
41.401	37.982	134.794	89.291
77.531	34.522	81.626	50.436
59.62	20.892	71.567	59.62
70.882	13.186	75.838	48.216
95.287	8.462	132.901	74.974
62.319	29.585	163.433	66.082
49.841	30.328	168.761	105.287
43.285	34.423	61.561	104.361
52	27.24	62.742	68.707
27.721	23.105	41.583	76.407
44.158	27.539	39.719	94.684
32.655	24.654	53.402	92.4
42.251	39.072	53.721	94.405
60.941	58.113	69.704	101.742
48.058	59.684	76.297	93.628
29.031	67.355	59.821	111.069
41.27	82.323	74.484	100.667
31.177	63.948	74.745	100.735
21.824	39.999	82.783	104.63
61.841	51.273	26.034	101.502
32.554	54.262	36.169	105.127

	38.744	80.343	29.403	50.193
	47.768	58.596	39.629	53.263
	45.835	61.704	42.073	37.448
	39.285	59.791	23.55	42.155
	42.145	45.924	32.111	41.131
	38.419	27.247	36.68	27.191
	43.95	48.444	34.799	28.225
	38.508	40.124	35.071	26.987
	50.08	54.687	43.301	25.765
	42.812	68.975	24.994	34.063
	40.75	64.984	30.833	16.895
	19.822	117.874	32.467	30.909
	30.822	69.094	26.45	31.508
Mean		**49.07**		**71.768**
SD		**21.759**		**36.451**
SEM		**1.777**		**2.976**

Figure 4. Day 12 1h in subjective night

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
82.298	141.567	219.662	134.099
100.33	98.031	193.153	153.308
77.271	66.476	205.89	143.676
51.965	97.937	223.542	131.565
105.155	77.667	201.058	97.703
95.265	94.574	232.532	106.031
109.265	65.817	213.5	116.2
109.072	69.971	168.756	114.862
101.499	71.851	171.494	141.339
90.562	89.031	123.427	199.937
74.978	69.813	185.8	172.778
102.28	62.897	115.133	127.57
124.666	92.066	236.034	132.681
86.987	55.287	201.826	148.597
63.913	42.145	188.647	152.172
92.142	59.266	195.112	144.196
54.126	142.296	219.183	119.622
84.085	46.692	185.641	100.937
47.585	47.182	139.482	131.306
75.467	75.981	153.015	124.516

134.958	69.287	129.773	149.331
75.004	70.163	192.704	137.523
69.446	88.777	121.609	119.148
90.32	64.448	176.395	101.872
88.527	81.564	167.711	153.887
68.307	108.003	190.271	174.369
52.164	78.638	138.666	163.602
34.689	85.951	118.625	215.497
105.62	28.251	131.621	187.595
122.434	97.471	142.577	204.56
102.704	33.768	221.17	212.425
104.43	97.968	202.68	215.625
62.605	95.629	218.183	227.089
44.279	74.349	231.906	225.511
70.342	165.179	247.972	216.005
98.722	147.992	201.64	207.667
32.737	104.475	238.794	196.567
46.793	98.735	149.637	157.06
104.242	89.893	182.164	181.728
51.545	55.271	230.532	215.467
74.642	87.539	159.633	214.809
98.798	113.784	146.764	238.305
79.323	88.763	181.839	222.403
48.792	67.949	180.864	182.35
51.051	81.534	212.825	247.724
34.434	126.85	213.287	230.449
42.329	86.33	195.485	216.438
31.715	53.187	206.239	226.045
56.227	80.589	199.944	226.326
30.884	35.125	150.845	187.929
59.132	114.887	168.455	239.543
51.944	117.181	105.267	151.766
65.788	83.877	83.865	223.691
42.373	150.094	87.306	217.596
56.996	184.479	96.569	187.194
66.658	86.461	172.372	230.603
66.823	88.439	118.119	234.793
32	81.164	129.347	182.66
29.188	62.317	108.723	117.755
37.249	66.499	128.958	139.088

	41.197	27.827	116.093	122.749
	62.235	43.534	143.295	155.633
	46.403	86.696	171.769	152.832
	47.234	65.931	72.502	105.794
	43.196	85.649	112.561	104.637
	38.01	70.27	95.067	104.527
	48.805	89.2	236.783	117.586
	34.175	87.038	183.935	112.262
	40.214	92.814	224.864	113.253
	57.788	84.459	145.82	155.798
	32.963	78.42	130.113	135.759
	37.547	51.423	129.441	152.548
	91.8	48.639	122.77	141.311
	90.058	66.814	167.484	129.168
	73.591	80.112	86.696	155.731
Mean		**75.657**		**166.841**
SD		**29.316**		**44.425**
SEM		**2.394**		**2.627**

Figure 4. Day 12 7h in subjective night

Cytoplasm Pixel Intensity		Nucleus Pixel Intensity	
53.347	172.083	51.775	143.324
73.665	123.686	58.267	132.342
78.535	186.449	71.207	128.888
62.347	167.659	76.642	233.283
59.474	151.529	89.28	227.477
48.64	101.929	99.02	248.265
34.191	99.491	143.897	228.854
55.757	115.866	75.375	245.88
52.601	198.943	84.215	213.185
66.01	178.064	90.071	209.526
38.239	149.512	121.501	189.601
41.165	127.37	93.725	221.503
60.911	89.383	87.717	234.16
53.609	151.816	110.53	209.316
61.911	47.302	141.23	222.994
68.662	62.094	143.702	232.676
62.456	51.421	124.182	202.405
39.987	72.465	70.391	218.797
26.339	46.114	80.956	169.566

21.729	51.298	60.526	208.78
29.665	96.787	54.671	200.603
58.841	75.509	63.815	217.172
47.453	64.663	38.699	211.439
60.018	73.317	44.29	201.29
52.126	72.927	160.732	167.954
73.733	47.795	180.632	186.534
50.126	36.274	161.808	174.647
44.771	49.274	103.815	192.174
80.499	52.276	103.176	238.096
78.799	81.485	75.44	178.158
59.968	46.329	118.5	200.962
93.871	56.524	110.379	159.935
75.885	49.626	138.191	201.645
58.302	48.141	123.952	234.47
73.529	58.751	118.025	230.095
60.777	58.027	160.472	225.572
53.889	89.663	122.619	247.295
35.273	115.756	104.088	229.513
153.771	158.528	113.257	214.263
189.406	94.027	117.201	226.134
143.014	79.59	114.063	217.326
133.618	53.726	117.587	170.678
170.422	64.164	194.619	131.604
148.194	126.662	145.156	170.83
136.011	158.559	140.025	184.943
88.317	149.797	165.69	148.143
143.35	118.561	153.629	155.08
117.381	161.735	143.302	162.174
123.227	118.828	171.906	161.445
96.194	80.728	198.369	179.023
113.056	91.621	174.368	133.974
90.521	102.033	166.334	128.922
107.34	54.505	120.775	133.916
89.92	61.029	159.113	104.79
122.639	112.317	147.824	166.197
129.954	127.97	162.358	137.086
152.198	154.009	133.038	161.102
109.852	142.571	115.043	153.751
149.646	106.332	119.232	180.395
126.627	112.827	124.046	153.473
120.064	137.762	121.623	161.722

	196.662	118.61	144.87	103.354
	211.49	126.207	110.847	96.58
	212.359	130.528	167.878	175.708
	143.668	107.484	186.726	165.605
	148.434	87.388	181.887	163.224
	83.379	94.444	134.648	158.619
	113.087	143.009	104.537	135.421
	215.435	165.714	99.725	156.952
	143.144	150.022	129.183	124.225
	150.658	129.289	109.698	142.779
	183.318	178.896	164.08	122.123
	161.205	173.051	135.85	148.15
	125.154	143.759	116.669	178.993
	175.455	169.899	131.99	134.262
Mean		**102.46**		**151.04**
SD		**47.218**		**48.556**
SEM		**3.855**		**3.965**

Figure 4. Day 12 1h in subjective day

Cytoplasm Pixel Intensity		Nucleus Pixel Intensity	
73.667	44.443	109.895	105.677
65.703	63.289	93.798	135.563
56.141	56.039	126.795	109.035
28.542	62.22	157.123	82.431
26.298	58.093	156.372	78.994
24.36	75.478	164.192	84.601
43.23	31.07	87.689	85.666
59.482	30.822	94.618	94.553
53.734	40.23	96.367	72.351
113.676	44.649	139.912	100.5
144.963	42.841	111.068	79.543
103.167	51.655	136.499	67.199
10.393	44.96	64.267	103.165
14.537	46.908	62.067	73.522
14.612	42.287	66.255	86.526
35.027	102.526	130.652	87.174
45.672	129.754	108.392	94.13
34.045	128.191	100.086	77.303
20.329	91.005	96.011	84.218
38.139	78.782	108.788	81.328
79.753	77.527	80.127	84.47

50.285	101.828	184.858	66.997
71.842	58.603	186.255	78.073
21.068	84.085	147.563	64.165
63.006	79.602	119.67	113.727
37.919	72.365	165.565	110.676
57.438	20.322	178.834	104.381
37.59	59.682	102.059	130.373
33.133	59.07	136.695	114.212
37.194	70.12	85.439	125.143
34.46	49.705	65.959	103.836
35.714	60.341	107.789	94.741
33.025	25.184	132.064	83.914
56.947	34.868	197.901	104.667
15.994	54.078	151.952	104.065
45.845	60.615	137.679	100.618
11.593	60.8	197.535	99.506
70.436	29.924	138.615	77.538
27.399	59.784	106.764	92.407
81.807	64.914	146.053	92.927
55.454	46.736	183.162	79.666
50.166	40.181	172.562	101.021
39.081	55.005	194.383	147.186
18.62	56.008	118.511	172.131
26.933	40.86	166.059	123.055
22.545	30.222	81.253	144.514
22.925	83.388	113.49	138.382
75.807	74.963	110.853	140.478
70.883	58.34	138.639	133.153
79.945	69.609	142.119	108.029
30.034	53.779	132.765	97.19
111.468	62.827	122.751	202.199
48.767	63.789	127.658	193.276
30.38	59.075	112.854	194.323
39.019	97.483	173.342	131.727
67.514	138.46	89.562	185.768
61.116	75.815	172.19	136.274
77.484	122.281	129.597	90.915
58.49	44.313	144.208	95.217
65.966	52.787	152.64	102.717
59.603	56.329	115.808	98.27
101.294	115.776	103.046	90.655
67.602	78.925	91.925	92.607

	63.833	53.349	118.064	94.181
	59.957	52.695	106.933	121.086
	32.887	78.399	98.405	108.783
	71.295	63.201	138.05	161.631
	69.999	64.975	154.681	179.834
	77.454	46.56	99.366	180.154
	104.403	63.456	92.183	123.611
	80.649	64.798	113.881	105.93
	90.177	131.404	92.365	124.229
	37.229	135.627	82.147	158.513
	48.834	93.673	89.48	158.122
	48.765	111.361	108.038	164.653
Mean		**59.732**		**118.484**
SD		**27.879**		**34.452**
SEM		**2.277**		**2.813**

Figure 4. Day 12 7h in subjective day

Cytoplasm Pixel Intensity		Nucleus Pixel Intensity	
74.25	23.24	168.8	135.37
110.775	16.038	169.334	123.542
86.517	9.868	151.23	105.112
79.387	12.855	174.829	175.777
83.909	9.524	139.907	160.795
77.955	9.524	129.006	132.308
28.322	12.218	130.075	134.222
37.388	14.142	137.777	156.528
49.067	32.556	96.313	91.478
44.912	91.082	179.447	103.917
64.11	93.366	169.936	162.667
64.801	68.391	162.215	119.929
79.384	100.535	135.517	216.812
81.774	102.233	108.167	245.47
89.972	139.414	136.443	252.764
66.475	134.632	133.139	251.316
66.313	118.322	179.062	252.065
101.896	161.25	230.25	248.296
72.057	195.171	130.918	173.855
70.649	175.695	119.271	228.177
63.652	165.016	126.032	137.005
53.773	93.547	171.25	112.65
72.925	184.853	97.134	154.811

73.148	180.765	179.423	150.192
107.871	88.068	166.867	131.335
58.665	190.103	116.748	122.569
66.211	166.064	161.108	94.117
82.309	189.946	104.004	142.075
74.95	189.425	135.801	98.697
62.273	169.511	156.769	159.761
132.567	158.048	124.746	169.201
78.441	127.991	114.005	95.632
54.648	134.905	123.568	115.188
111.325	112.429	180.561	238.924
67.675	62.033	60.698	239.018
68.837	120.225	137.711	200.411
70.306	113.417	137.229	251.113
114.551	106.771	118.257	254.005
89.446	53.654	104.618	242.823
76.378	77.428	136.742	233.38
94.05	55.896	146.835	244.819
73.537	69.277	98.083	205.919
157.255	98.161	100.111	138.272
89.403	73.422	126.623	152.092
87.587	66.985	147.839	118.427
75.045	68.266	141.48	35.902
81.565	8.792	171.461	29.479
64.544	16.027	113.413	25.823
29.635	15.569	99.391	35.392
76.642	3.621	86.436	33.896
14.345	10.131	118.112	19.575
10.857	4.078	160.994	28.894
39.831	16.131	199.328	18.287
6.657	35.241	156.181	27.144
5.274	17.728	111.074	71.12
9.66	16.076	146.005	73.047
20.111	15.182	138.763	85.404
20.067	13.569	141.935	177.273
13.308	16.414	134.871	139.951
24.875	13.065	127.78	134.226
10.234	10.235	115.58	186.513
11.581	16.266	85.146	157.082
29.133	14.662	81.4	97.897
17.114	14.823	84	29.842
12.816	15.291	94.999	38.765

	16.165	19.658	108.982	32.87
	13.952	9.358	113.883	33.957
	14.605	11.5	83.059	26.601
	7.877	25.067	100.404	32.873
	16.338	18.626	109.765	32.8
	10.594	10.883	159.473	37.409
	20.687	9.59	107.244	26.198
	16.174	9.924	83.578	28.606
	18.298	9.147	105.176	35.146
	9.594	62.818	97.287	26.148
Mean		**62.167**		**128.417**
SD		**50.641**		**58.423**
SEM		**4.135**		**4.77**

Figure 4. Day 13 1h in subjective night

Cytoplasm Pixel Intensity		Nucleus Pixel Intensity	
32.903	88.196	134.281	250.447
53.966	80.564	141.319	202.567
36.713	71.925	144.52	216.149
25.505	71.504	91.489	192.578
44.518	118.598	91.161	178.588
49.626	122.287	84.734	194.018
31.086	113.702	121.978	239.074
43.496	98.713	145.898	236.582
72.341	116.381	103.429	214.345
36.891	75.766	83.717	124.699
59.065	49.382	98.556	107.032
73.762	48.104	79.761	142.348
80.202	30.497	104.652	113.257
89.806	44.086	99.158	109.114
87.169	44.458	99.371	104.8
71.988	41.029	111.393	217.63
34.011	12.104	106.918	186.784
60.749	16.279	142.85	207.543
47.539	17.304	139.566	112.403
35.7	32.579	222.174	149.581
32.559	30.08	203.791	141.849
42.898	26.217	101.746	199.192
84.343	62.489	88.387	162.456
49.568	77.87	146.548	209.178
70.629	58.34	166.051	234.654

55.379	71.939	162.707	173.528
94.742	56.368	160.971	177.962
109.845	59.656	211.878	245.092
119.623	26.188	211.774	251.019
85.172	29.141	230.4	185.083
63.731	58.98	102.369	141.827
87.816	74.677	170.413	144.622
75.167	60.099	124.341	137.227
59.987	65.616	117.01	150.114
87.262	46.205	139.626	153.708
81.753	75.617	178.876	155.241
102.825	44.028	120.107	157.958
61.916	33.257	92.526	179.75
117.278	44.278	104.216	186.088
88.631	62.968	134.333	115.34
142.482	48.813	134.474	129.6
94.808	39.085	129.876	126.549
68.852	40.827	137.594	97.261
99.391	80.517	199.204	80.734
69.903	75.777	177.563	108.092
121.507	32.176	167.094	98.917
98.878	37.229	114.296	95.298
183.16	110.129	144.513	104.785
104.721	95.437	110.05	155.263
99.909	94.046	128.987	155.786
102.994	60.931	91.548	168.077
150.049	60.455	124.379	115.167
82.034	83.892	104.208	102.637
118.195	91.793	92.032	119.729
161.836	66.127	249.429	150.72
103.719	82.544	94.504	199.115
137.201	62.237	132.852	136.756
126.568	76.405	161.457	170.823
78.658	62.975	152.911	166.243
66.745	75.681	155.088	169.415
105.966	103.433	146.193	139.478
99.613	66.039	112.255	185.107
100.012	62.995	152.31	105.04
94.621	54.537	228.201	128.491
95.19	39.654	245.383	119.016
61.93	92.105	234.696	122.591
112.185	96.389	234.937	150.261

	99.104	66.734	242.342	143.308
	91.443	72.428	251.319	144.86
	92.742	98.212	232.232	120.575
	104.996	84.721	238.714	94.561
	110.146	94.771	227.426	184.344
	73.882	68.679	220.033	121.07
	74.516	72.893	232.099	75.263
	95.68	100.176	217.231	145.615
Mean		**74.467**		**153.096**
SD		**30.473**		**47.47**
SEM		**2.488**		**3.876**

Figure 4. Day 13 7h in subjective night

Cytoplasm Pixel Intensity		Nucleus Pixel Intensity	
62.154	28.671	150.692	153.956
42	33.894	118.67	103.934
50.83	37.345	128.714	115.874
35.931	61.56	100.826	118.415
41.393	46.336	96.842	78.974
47.49	50.498	101.311	106.304
43.942	61.92	107.779	140.912
46.546	21.804	118.625	163.844
45.167	53.842	146.708	108.333
76.071	31.11	87.949	53.921
69.945	56.837	109.965	69.34
29.497	45.237	85.914	64.606
35.649	41.903	120.263	160.906
47.122	55.8	115.184	195.003
63.003	57.011	100.817	131.509
34.678	56.43	159.184	91.922
84.694	44.864	135.444	92.892
38.777	50.512	141.817	91.618
32.869	34.596	100.386	61.07
93.542	52.25	115.647	62.163
67.77	50.98	114.709	57.604
87.487	47.324	92.325	61.152
64.988	36.507	111.735	70.205
80.715	43.691	117.878	61.867
63.848	48.901	132.578	82.339
82.791	47.974	118.701	109.422
66.388	35.088	145.663	66.137

53.507	39.497	161.366	131.127
48.104	31.662	128.868	103.419
43.516	35	140.868	114.413
59.144	12.928	133.072	47.987
45.32	67.26	107.455	59.125
51.091	58.594	160.807	54.581
41.437	77.213	112.842	112.444
42.847	63.692	144.544	93.831
56.391	58.143	86.059	64.98
56.689	76.745	119.85	74.176
61.829	112.452	113.667	69.857
57.192	108.79	128.312	74.552
42.944	63.489	168.284	94.022
47.824	45.45	124.493	102.199
51.879	55.171	113.635	72.265
48.011	102.849	131.835	122.604
68.723	70.602	151.098	184.954
40.999	67.734	166.15	127.274
45.284	97.066	153.5	45.819
50.42	91.91	146.221	58.036
44.078	70.18	122.926	52.004
36.721	115.466	131.817	67.38
60.05	70.758	138.964	87.103
74.56	113.101	116.333	86.388
46.766	57.085	127.691	94.648
44.424	101.61	112.676	77.495
64.18	64.35	91.925	78.817
56.506	37.397	102.686	73.542
37.419	70.941	158.6	88.262
37.701	48.715	164.734	89.268
51.389	73.524	107.117	103.239
21.754	91.179	96.301	102.127
35.008	34.7	114.711	89.528
28.699	34.654	136.87	85.579
59.697	20	126.898	105.275
38.212	51.921	124.206	53.899
40.815	41.49	149.662	76.167
22.886	35.134	135.228	112.165
24.388	60.721	156.659	85.164
37.437	25.207	134.867	86.735
34.474	60.833	128.958	112.515
38.56	59.642	110.545	102.767

	15.963	40.446	219.488	99.666
	50.997	75.717	170.985	81.188
	36.57	62.106	212.088	107.096
	37.068	92.637	184.197	73.12
	23.635	94.367	235.446	77.634
	39.62	61.21	120.553	74.133
Mean		**53.482**		**111.381**
SD		**20.439**		**35.629**
SEM		**1.669**		**2.909**

Figure 4. Day 13 1h in subjective day

Cytoplasm Pixel Intensity		Nucleus Pixel Intensity	
39.285	13.62	139.124	31.883
27.308	7.201	185.898	32.558
30.92	10.278	170.732	23.556
39.838	6.817	137.891	32.946
20.431	9.942	126.386	27.863
19.883	17.62	131.749	31.241
11.662	72.17	175.331	42.673
22.391	78.129	177.431	43.444
19.478	66.913	147.851	34.728
30.729	70.492	232.988	33.449
24.788	46.634	162.759	34.301
20.657	42.462	129.482	33.789
18.79	41.873	123.663	69.727
19.191	47.812	175.765	85.178
16.995	51.846	142.105	81.954
21.103	58.597	118.295	117.692
19.607	63.079	151.27	105.783
18.048	54.703	149.05	158.21
22.432	74.767	147.66	147.876
25.577	75.573	112.052	155.549
17.863	86.737	163.656	129.131
15.805	75.945	148.255	107.705
19.437	68.172	176.149	128.311
18.643	89.97	144.302	93.173
15.915	69.803	130.522	251.35
23.486	47.56	192.416	207.751
20.028	66.012	161.227	196.342
17.617	86.891	88.873	224.357
19.475	69.156	103.489	145.632

20.791	70.298	115.087	206.647
18.278	61.337	135.202	178.066
16.811	56.095	172.569	173.591
18.805	59.09	173.318	156.623
33.655	61.938	138.707	153.087
39.304	54.763	127.915	155.045
32.541	80.13	122.58	178.263
35.323	59.191	124.633	132.545
16.623	57.04	169.597	177.928
16.912	58.539	205.691	182.578
20.047	78.828	126.032	222.44
22.616	100.568	94.39	181.436
58.597	86.802	111.435	203.913
62.722	75.089	127.837	194.903
109.419	71.067	121.75	124.995
53.671	69.95	145.697	100.678
53.158	74.591	109.204	110.294
81.711	69.338	103.449	94.76
69.934	76.222	97.472	98.642
93.127	62.651	140.792	176.596
86.958	84.842	129.764	180.128
103.709	66.015	170.399	170.934
26.411	45.066	101.801	136.407
30.493	44.918	111.181	164.092
41.357	54.434	109.504	155.671
67.212	47.333	30.353	72.47
57.459	12.369	28.909	66.804
55.849	9.218	27.558	71.405
69.29	19.146	30.874	125.72
66.084	19.6	37.07	137.083
35.33	17.756	31.017	131.441
96.363	34.643	26.957	177.971
64.164	26.661	34.344	126.253
36.272	30.107	33.05	99.946
18.815	35.2	24.524	90.552
55.878	25.718	28.683	104.438
52.508	28.815	30.762	82.739
78.251	30.395	31.36	135.553
75.482	79.333	21.344	115.694
28.877	97.897	28.046	185.565
52.386	66.768	15.417	117.465
21.4	70.877	16.003	114.713

	11.597	41.75	18.494	99.956
	19.562	90.952	19.827	62.183
	15.772	86.186	20.02	59.889
	12.789	81.821	22.28	63.893
Mean		**46.625**		**114.996**
SD		**26.319**		**57.605**
SEM		**2.149**		**4.704**

Figure 4. Day 13 7h in subjective day

Cytoplasm Pixel Intensity		Nucleus Pixel Intensity	
104.093	15.98	114.032	39.95
41.5	15.361	150.186	56.248
102.749	14.4	87.672	53.685
89.708	15.97	102.944	117.725
86.916	12.784	106.816	109.073
88.642	20.975	95.514	95.696
128.278	40.2	102.627	107.252
113.686	43.654	110.04	111.652
89.722	19.62	130.574	110.281
90.157	23.235	181.575	85.439
108.132	33.009	178.803	111.007
83.129	39.418	194.572	117.623
121.693	50.15	133.3	90.251
83.886	31.532	108.988	107.338
77.974	49.183	124.753	71.941
99.166	35.727	184.202	85.309
90.494	44.553	155.231	112.138
86.152	61.29	150.059	80.636
139.415	61.788	142.795	134.206
162.938	46.611	182.287	120.575
59.02	31.705	184.011	162.044
136.932	28.194	127.12	124.365
58.084	27.738	158.79	116.518
110.876	7.358	140.776	146.25
74.079	12.414	171.6	95.349
74.062	31.262	226.378	67.125
76.651	29.08	193.777	64.817
193.349	32.511	141.321	123.152
62.171	9.007	55.211	83.525
114.352	9.198	102.913	94.37
82.041	31.32	127.227	96.488

81.6	11.23	121.401	103.192
152.14	26.728	161.427	55.972
78.82	20.873	120.418	109.727
55.417	41.58	119.341	124.246
47.807	18.322	121.966	78.803
121.61	35.572	203.023	113.167
78.629	37.171	82.276	156.288
81.329	55.089	160.076	133.66
39.521	46.909	139.232	100.5
64.776	63.825	101.663	99.283
30.933	54.582	138.82	101.81
62.155	70.019	159.575	59.226
13.146	41.258	175.886	105.278
23.24	31.536	130.884	81.162
14.746	38.117	136.364	126.504
12.978	18.883	163.047	104.76
10.354	57.52	108.939	134.904
11.623	48.754	193.668	70.994
14.245	53.449	177.525	58.048
15.898	64.612	117.654	50.66
30.299	50.978	211.192	167.444
17.306	88.362	239.501	168.385
17.816	83.093	168.414	120.529
12.873	57.395	207.347	31.7
12.259	48.251	119.914	32.053
15.309	44.167	155.045	27.519
16.449	49.573	123.125	35.588
15.955	60.984	153.689	29.103
20.723	40.647	133.544	30.909
29.514	33.356	57.665	19.011
17.306	62.403	99.19	26.918
18.42	43.038	72.692	24.271
14.59	57.204	74.094	107.376
9.981	75.357	51.959	102.711
11.426	112.283	55.299	92.205
16.577	93.063	37.2	144.284
11.987	118.344	37.603	129.2
11.63	89.882	28.933	114.944
9.112	75.346	47.983	36.514
16.053	68.551	53.997	26.4
3.528	75.079	53.185	27.77
12.722	33.397	63.25	22.053

	13.089	56.631	64.752	13.391
	13.699	85.395	54.699	29.342
Mean		**51.824**		**107.889**
SD		**37.099**		**49.136**
SEM		**3.029**		**4.012**

Figure 5. Day 11 1h after off

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
64	101.154	187.895	72.855
61.75	105.145	157.782	96.046
64.153	135.82	136.058	120.282
68.722	157.91	122.844	109.589
59.491	123.565	170.153	119.974
73.751	98.306	160.304	169.306
45.623	95.507	175.491	133.559
90.656	77.121	181.542	166.518
55.012	65.453	141.988	152.414
77.359	94.93	134.392	139.221
69.541	45.105	129.849	181.009
79.421	35.861	128.221	92.615
105.66	37.701	136.106	85.354
87.264	168.131	139.847	100.925
72.108	147.92	123.437	78.545
113.586	43.831	125.511	84.316
65.732	33.534	128.629	88.561
83.918	53.202	149.754	129.679
83.367	59.176	158.57	122.168
103.019	82.526	151.269	117.269
87.096	86.068	158.762	206.965
53.134	20.689	147.422	187.618
78.633	35.747	161.961	150.192
73.682	91.971	127.101	166.009
74.901	126.681	164.771	145.814
92.317	35.808	162.392	162.536
83.232	40.809	180.035	166.25
74.465	70.844	151.688	204.911
80.688	65.659	175.741	164.014
86.929	68.705	157.342	153.019
73.85	57.724	141.571	158.483

79.189	46.694	173.984	167.183
84.671	57.663	155.596	217.476
79	15.444	163.966	202.596
92.264	159.09	180.396	230.091
91.486	98.814	146.404	161.228
78.253	119.811	158.139	176.776
98.59	67.827	151.246	148.51
56.441	87.95	126.268	211.499
70.316	87.052	189.632	199.427
47.709	100.134	158.808	157.345
38.372	81.112	133.277	216.891
41.539	114.035	96.632	226.852
75.596	46.44	74.361	183.142
91.686	72.618	71.907	204.713
71.899	98.601	97.809	219.633
84.132	69.047	134.143	242.745
76.646	70.608	110.345	207.493
60.39	100.254	94.545	231.139
66.264	86.969	103.961	231.598
75.683	48.559	90.743	210.411
99.244	51.096	111.264	205.264
89.808	62.637	130.066	188.583
80.949	70.241	144.909	200.763
82.349	64.319	102.783	196.171
78.727	33.995	112.082	205.684
73.504	45.465	93.191	172.882
50.093	66.691	142.921	161.255
76.21	65.499	115.23	159.888
64.044	48.101	108.372	185.897
104.952	35.055	172.875	159.868
130.117	32.347	160.727	161.679
101.554	25.865	163.415	184.571
106.523	44.728	188.311	185.247
96.695	19.783	150.461	153.022
106.694	23.153	147.968	162.806
72.26	49.002	141.598	215.54
88.288	39.869	143.667	192.322
101.447	64.012	129.6	140.179
148.54	46.36	127.226	153.834
78.498	61.222	124.128	158.235

	46.096	49.902	112.725	211.962
	108.882	74.978	118.464	220.413
	102.219	120.743	125.759	204.657
	57.259	67.952	90.666	210.04
Mean		**75.616**		**154.017**
SD		**28.49**		**38.36**
SEM		**2.326**		**3.132**

Figure 5. Day 11 7h after off

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
72.325	74.744	234.643	199.774
40.167	72.001	243.095	180.144
87.59	116.459	195.099	153.809
78.599	68.662	181.98	184.426
119.699	108.773	219.535	220.643
143.322	126.707	181.367	161.978
84.396	59.932	213.125	199.64
28.575	81.653	242.837	168.824
47.925	126.433	178.791	181.392
86.67	91.176	252.474	231.265
115.328	48.424	249.793	227.477
55.16	100.549	232.563	241.601
106.627	42.568	200.584	181.9
140.369	74.054	247.956	184.011
117.136	85.716	232.881	167.554
44.176	114.078	179	92.461
108.809	53.069	167.901	182.777
115.958	71.9	153.039	179.229
85.887	142.455	175.44	179.16
79.235	150.667	168.374	152.84
115.044	173.423	194.123	132.896
118.596	117.701	174.899	206.099
63.304	155.222	200.951	225.618
60.289	118.294	168.436	222.759
57.268	133.184	176.9	190.251
74.443	144.078	158.086	161.33
71.503	152.844	205.445	160.618
143.756	151.679	190.249	197.039
155.372	148.865	222.02	160.548

123.194	157.318	176.827	150.113
101.624	175.333	150.616	229.885
54.277	94.18	151.325	212.016
82.315	126.974	139.7	175.141
28.588	100.08	164.054	218.233
91.268	95.444	169.414	222.386
65.454	42.659	181.874	193.167
73.693	71.83	190.544	116.8
59.774	117.799	183.281	106.781
92.073	58.683	188.474	165.096
104.657	49.42	150.45	114.807
51.329	164.733	142.715	160.108
125.792	124.92	156.739	209.053
179.407	95.95	160.025	178.457
111.873	95.106	142.906	148.89
102.561	70.901	144.914	169.642
107.353	43.194	142.348	187.488
125.665	81.185	153.931	144.396
140.576	72.108	163.673	123.051
130.901	48.071	181.232	157.153
86.94	36.288	185.967	121.408
82.274	57.83	149.778	96.686
67.401	66.568	148.365	131.308
80.23	56.148	172.993	121.316
121.314	96.781	167.286	96.24
110.132	62.474	113.969	72.766
98.92	63.483	121.305	122.012
86.633	97.447	95.306	113.823
141.975	38.579	175.357	173.929
77.154	54.759	159.638	188.591
78.027	95.12	156.893	181.651
90.655	59.707	167.312	152.898
89.39	74.629	195.993	150.459
81.804	81.585	181.921	121.716
117.298	54.437	229.543	116.636
77.432	51.783	217.166	162.057
78.545	52.501	209.267	150.997
74.626	109.461	223.634	168.78
46.207	54.89	220.493	135.708
66.175	35.89	162.713	194.059

	90.868	47.273	179.402	185.606
	79.704	22.701	168.607	174.853
	86.837	76.522	208.969	162.274
	58.59	60.907	210.904	141.982
	65.09	82.688	209.217	125.047
	96.747	85.907	214.677	114.245
Mean		**89.79**		**174.074**
SD		**34.365**		**36.605**
SEM		**2.806**		**2.989**

Figure 5. Day 11 1h after on

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
62.6	79.023	111.68	116.752
46.446	87.601	138.933	121.755
67.603	26.943	101.075	123.824
59.909	60.323	137.736	121.68
37.419	19.035	123.679	88.951
43.428	49.171	117.09	110.212
66.821	65.657	83.373	59.926
35.678	63.283	116.851	149.534
30.758	59.85	79.317	125.814
17.508	119.369	86.017	88.932
53.202	60.1	68.382	97.283
41.057	69.966	64.726	89.538
63.979	50.856	81.048	104.535
60.919	22.012	62.565	157.779
60.173	34.315	68.187	126.153
87.046	29.401	86.331	112.51
91.312	51.052	120.201	143.122
79.423	39.592	93.298	114.449
81.22	26	228.388	103.539
42.61	30.659	178.919	135.871
54.777	81.332	230.434	145.997
61.409	68.127	192.71	109.179
61.296	66.283	219.569	155.628
60.266	85.075	186.43	110.379
86.515	70.604	101.076	82.194
75.738	69.364	110.105	50.403
95.873	64.751	112.863	60.951

37.149	53.776	98.348	77.723
54.561	100.459	87.449	104.319
97.727	122.343	70.174	112.938
128.576	49.624	96.713	151.559
62.174	125.824	108.327	91.23
58.152	176.216	102.072	90.335
70.766	63.96	100.258	143.893
44.821	93.342	102.753	115.306
38.52	140.101	111.003	157.807
50.168	79.793	91.485	77.335
46.725	56.857	133.459	94.869
33.519	73.372	102.567	111.244
35.056	46.629	71.503	143.353
32.173	73.329	73.418	143.132
34.213	51.773	68.672	141.566
24.184	93.014	90.698	95.931
39.465	71.371	81.912	77.579
48.625	65.566	90.455	93.631
43.712	63.184	116.505	137.257
34.365	35.057	102.707	83.701
58.539	24.689	91.006	93.72
31.788	37.734	110.648	47.889
38.393	28.8	124.217	92.898
74.779	36.94	135.743	57.951
45.554	35.51	39.073	68.648
43.1	14.249	31.867	89.451
43.108	40.544	20.691	53.594
63.052	14.446	219.282	65.587
52.245	37.841	137.544	56.195
78.064	71.092	124.783	53.904
50.7	24.216	89.735	117.307
44.773	27.84	58.16	123.439
86.262	36.96	62.745	96.357
27.005	16.861	74.704	157.419
87.137	48.571	64.105	128.318
43.986	74.906	87.256	133.768
58.709	41.74	79.495	132.674
77.808	74.648	105.452	111.169
40.414	48.994	79.229	118.926
43.693	24.143	108.306	100.896

	54.79	33.061	90.154	79.323
	81.66	50.226	75.74	135.162
	33.662	62.815	76.314	50.333
	57.757	60.523	89.356	56.71
	80.182	79.589	124.381	59.164
	62.668	30.295	108.891	97.154
	144.232	37.056	106.918	104.73
	85.293	56.072	72.434	65.476
Mean		**57.898**		**103.93**
SD		**26.656**		**36.44**
SEM		**2.177**		**2.975**

Figure 5. Day 11 7h after on

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
61.714	37.329	77.434	54.25
58	36.958	60.8	87.197
70.423	32.914	83.834	77.116
37.755	28.397	77.955	79.686
44.366	33.317	64.773	56.148
62.024	50.618	85.474	79.055
37.628	31.549	83.582	94.443
29.186	38.578	71.804	75.156
26.541	18.468	75.109	76.583
25.576	45.714	67.767	94.673
19.379	47.981	72.54	71.844
33.902	36.455	58.787	82.439
42.785	44.663	67.757	87.072
32.764	29.929	63.674	68.008
46.65	49.109	55.416	74.085
46.742	55.822	57.388	116.772
22.789	60.266	68.148	71.244
40.483	33.357	86.682	67.678
29.079	37.541	61.356	69.802
35.146	35.485	55.073	72.936
48.364	18.084	51.294	64.945
40.127	23.218	129.134	136.905
30.728	35.542	153.501	103.729
43.954	33.256	115.989	127.83
29.246	37.695	145.624	139.168

32.921	32.155	102.864	152.533
36.44	50.741	122.66	148.395
47.22	20.886	152.202	106.234
44.405	12.72	137.383	91.957
24.793	19.512	119.679	91.564
30.502	38.797	60.114	63.126
42.307	26.929	97.358	92.956
42.266	31.839	90.337	81.753
39.354	27.179	125.596	101.295
18.266	12.25	92.987	68.849
48.3	51.723	80.459	87.96
39.338	27.929	135.098	84.973
33.716	52.046	130.476	83.14
39.66	41.29	141.752	77.349
37.956	29.11	149.781	88.675
26.387	32.894	132.76	86.3
40.059	44.98	114.868	87.729
21.471	49.598	96.28	100.099
34.523	21.311	71.852	118.335
38.122	30.869	68.937	95.111
25.02	24.344	127.914	84.942
33.871	21.126	75.865	81.278
53.77	30.457	100.876	81.101
22.032	39.768	76.613	59.03
18.144	9.785	70.058	74.866
47.994	57.784	82.363	64.977
17.129	38.17	103.456	81.828
40.034	30.681	128.778	75.663
49	37.879	100.442	66.014
49.769	46.226	103.263	87.017
32.45	40.983	86.346	75.214
30.746	28.06	94.4	77.048
51.546	34.743	58.293	102.849
65.956	34.685	96.906	91.768
43.634	25.945	80.135	101.259
42.686	98.286	93.362	139.811
68.938	51.304	73.493	151.51
60.552	60.491	70.568	136.291
36.85	68.55	72.025	127.606
34.893	40.182	68.802	126.971

	32.346	44.626	69.769	118.359
	38.598	38.224	81.681	128.65
	30.801	46.655	82.061	113.931
	19.703	33.71	84.429	102.587
	32.741	33.129	76.65	71.096
	55.462	54.841	79.064	74.405
	31.863	44.966	83.294	61.005
	44.9	29.575	64.819	131.462
	32.269	33.766	79.568	132.223
	32.359	42.77	74.245	129.481
Mean		**37.987**		**91.608**
SD		**12.952**		**26.059**
SEM		**1.058**		**2.128**

Figure 5. 1h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
34.675	74.074	114.301	59.518
37.831	61.27	112.59	53.558
45.295	66.403	99.668	57.59
41.437	47.669	124.323	49.826
46.097	42.809	101.767	53.948
53.537	40.581	139.224	85.18
45.925	45.336	75.429	69.458
37.898	47.675	106.259	59.028
45.447	25.336	112.053	83.177
31.233	34.448	76.649	59.525
50.929	27.512	95.68	68.567
42.154	20.444	49.874	105.056
45.093	32.422	45.732	102.246
45.427	42.602	52.894	99.672
47.839	37.364	108.874	50.599
45.283	29.699	74.7	41.356
41.717	37.992	89.021	51.232
39.812	49.797	71.639	45.127
34.311	48.511	76.34	29.937
38.73	46.8	111.22	41.634
35.893	43.925	105.099	56.255
27.419	46.001	102.589	55.904
38.347	38.154	96.698	53.591

31.226	29.763	93.939	74.518
44.5	48.25	70.768	83.853
42.105	43.444	77.152	76.412
44.024	42.949	80.141	137.77
44.078	49.717	80.48	118.687
50.586	46.961	80.517	106.97
45.367	41.268	95.439	77.024
45.279	43.607	94.61	103.232
52.508	27.641	79.196	90.59
47.143	38.965	133.436	106.298
43.159	46.59	135.751	87.716
42.856	36.231	124.277	71.864
34.456	32.967	78.96	85.592
43.755	42.853	73.828	96.846
36.537	50.465	83.781	90.861
23.589	46.807	59.828	94.346
35.184	51.621	62.334	78.688
35.224	51.247	63.115	85.144
39.641	54.2	82.091	54.258
49.186	43.879	86.328	47.429
66.297	53.987	102.7	51.245
57.464	46.403	95.996	67.498
49.68	40.613	79.981	86.989
45.931	43.699	90.58	75.252
43.053	39.758	74.475	51.043
33.249	38.871	48.135	64.916
40.978	42.642	58.518	69.871
35.714	47.404	77.454	111.431
37.44	42.002	109.622	73.659
26.11	51.919	78.586	67.497
54.143	33.021	57.328	66.797
33.651	32.327	51.487	77.764
38.785	39.93	53.939	90.49
23.413	22.343	119.155	125.937
32	24.811	105.844	113.604
42.016	26.671	109.915	97.94
64.028	22.567	35.679	63.159
38.432	22.865	28.477	71.865
41.334	30.848	34.727	50.91
36.176	46.078	33.052	52.034

	40.478	34.202	48.455	53.837
	40.472	41.997	51.692	62.922
	33.166	46.817	78.292	82.019
	38.923	52.39	107.412	104.752
	49.046	68.715	97.244	87.201
	45.39	40.475	48.348	99.964
	32.652	76.535	45.028	70.544
	35.159	64.333	54.069	98.425
	37.051	67.853	74.867	90.253
	41.656	51.571	68.5	99.582
	22.752	56.909	75.262	93.871
	56.796	46.828	57.881	87.471
Mean		**42.332**		**79.281**
SD		**10.259**		**24.326**
SEM		**0.838**		**1.986**

Figure 5. 7h *in vitro*

Cell Pixel Intensity

83.295	80.433	64.742	22.553	45.611
163.354	47.867	36.78	47.702	35.624
107.224	41.729	42.797	40.425	50.038
51.982	72.233	49.112	41.219	43.473
48.302	63.464	89.549	43.675	33.33
45.087	74.226	32.499	51.317	28.845
37.553	35.852	76.783	40.868	46.729
61.456	66.099	48.957	46.271	48.68
46.106	82.888	31.855	51.96	66.882
84.531	44.034	50.542	47.725	38.788
66.364	96.715	42.217	54.304	42.758
72.944	73.252	54.68	41.759	42.294
65.282	44.752	75.203	59.677	40.088
72.375	51.244	37.776	65.069	47.072
62.363	53.5	27.626	57.754	44.458
116.625	54.136	41.598	26.482	26.816
67.518	49.594	33.531	67.33	36.079
55.648	45.095	41.149	47.253	55.685
58.674	54.935	66.673	44.45	64.122
139.209	24.432	34.514	47.638	61.503
72.676	20.133	49.992	57.251	55.176

57.5	75.814	58.858	69.942	33.799
65.507	58.759	40.841	42.243	44.041
59.696	38.107	52.914	24.873	47.783
59.908	31.876	55.115	39.561	48.433
99.682	46.511	72.522	64.945	45.705
94.636	54.592	77.005	41.718	47.999
51.291	39.728	57.858	65.216	46.038
59.131	63.326	51.817	49.41	49.722
32.81	57.126	52.244	34.732	40.945
			Mean	**54.285**
			SD	**20.228**
			SEM	**1.652**

Figure 5. 13h *in vitro*

Cell Pixel Intensity

22.274	20.366	16.223	18.85	24.395
23.125	28.706	18.62	20.93	24.172
21.147	34.759	15.443	17.811	23.856
11.57	27.441	17.752	16.441	12.86
17.465	27.982	26.769	31.501	13.291
23.985	33.181	17.079	18.362	14.69
19.407	15.032	13.116	18.881	13.059
20.75	16.525	15.416	21.375	18.04
23.396	13.488	14.653	23.636	21.788
15.224	12.916	12.899	15.454	19.589
18.324	17.011	19.704	20.475	16.891
15.415	16.285	23.974	17.7	20.506
20.655	16.139	15.513	51.196	16.837
13.945	21.719	15.013	19.529	20.938
24.524	13.718	19.399	14.407	10.283
10.803	15.047	20.072	16.779	12.416
13.141	13.864	20.166	11.962	14.807
14.557	12.441	24.813	10.346	24.389
14.398	13.198	25.051	15.845	17.356
20.554	16.62	34.759	22.263	35.383
19.905	16.827	23.539	12.97	23.504
18.042	17.112	21.89	9.364	34.275
23.648	14.39	19.314	18.028	14.339
12.871	17.91	21.637	15.935	14.042
11.097	21.368	27.782	16.568	19.787

22.962	14.603	12.329	13.01	22.127
19.636	12.592	38.689	14.629	19.905
21.001	15.232	21.645	14.45	20.623
16.491	12.521	21.213	14.357	21.903
20.26	17.81	19.56	18.561	12.52
			Mean	**18.944**
			SD	**6.05**
			SEM	**0.494**

Figure 5. 19h *in vitro*

Cell Pixel Intensity

7.471	11.654	6.655	15.692	8.691
7.415	7.827	26.41	14.803	4.88
13.69	10.45	21.164	16.215	11.599
9.743	9.732	19.414	23	8.392
9.901	12.829	17.787	9.71	7.271
8.796	8.765	18.684	9.411	6.123
9.301	6.757	21.077	9.267	7.813
12.115	9.814	24.29	7.573	5.642
8.801	14.185	19.158	15.732	8.138
13.052	21.225	22.928	24.215	7.221
4.692	29.722	29.043	13.028	6.665
6.556	15.928	28.317	19.45	11.93
5.471	14.136	17.035	19.153	8.514
13.738	20.286	19.117	21.433	27.443
16.358	22.118	18.333	18.77	21.083
12.413	17.942	18.641	23.824	20.594
7.144	16.815	11.229	16.438	16.027
10.324	26.242	12.25	20.106	23.303
9.977	31.162	20.321	4.934	31.865
12.885	18.796	21.934	7.809	17.086
13.044	18.3	19.41	8.582	19.385
6.205	14.008	21.905	7.126	18.798
8.222	8.073	25.253	4.937	19.583
6.205	6.518	18.428	6.9	5.666
5.363	3.566	28.738	7.13	5.844
11.198	4.728	23.02	8.032	8.458
11.877	9.832	14.094	15.13	5.958
13.743	11.738	18.537	9.529	4.703
7.048	7.738	23.179	11.833	8.524

9.69	4.951	21.991	9.106	8.07
			Mean	**13.872**
			SD	**6.841**
			SEM	**0.559**

Figure 5. 25h *in vitro*

Cell Pixel Intensity

16.39	10.379	16.416	20.301	20.387
13.42	14.89	19.294	24.119	15.631
9.702	7.656	22.26	25.397	14.914
45.753	14.739	14.841	20.745	18.106
11.482	9.788	19.074	27.04	13.213
17.311	10	22.221	24.045	17.121
22.114	17.185	23.066	19.941	10.266
14.048	16.649	14.509	11.562	26.071
29.056	17.577	16.197	18.036	16.408
18.952	18.385	24.702	21.496	19.13
14.306	16.756	16.05	20.096	21.994
16.614	36.058	23.877	29.796	17.62
30.05	11.819	15.887	12.637	11.328
25.968	15.117	20.292	18.342	11.406
15.952	18.265	20.114	16.713	18.177
17.173	15.658	8.955	16.904	21.335
20.027	16.481	15.57	42.943	25.821
22.642	22.639	13.007	20.143	20.553
15.305	15.523	13.313	23.077	12.523
18.442	17.917	23.428	20.115	12.949
26.295	29.244	24.116	26.561	13.599
26.485	16.813	20.916	21.695	9.409
15.415	14.83	14.291	13.285	19.569
27.703	18.976	13.845	15.939	18.836
25.815	28.154	16.719	17.936	21.857
49.456	15.419	16.572	13.375	20.79
29.848	27.428	19.876	15.705	19.968
35.109	15.197	18.447	13.429	13.98
12.121	20.118	18.285	16.968	12.045
13.351	21.086	19.412	17.037	11.275
			Mean	**18.962**
			SD	**6.555**
			SEM	**0.535**

Figure 5. 31h *in vitro*

Cell Pixel Intensity

19.331	20.31	19.572	15.964	15.656
14.25	19.47	25.413	14.053	19.112
12.338	26.873	16.671	16.658	24.194
27.371	14.501	15.087	20.841	20.758
14.415	19.517	18.73	24.86	24.209
18.947	29.693	20.326	16.79	21.348
23.977	26.965	13.547	13.707	15.971
32.357	17.284	19.033	16.217	19.003
13.835	19.508	16.104	18.467	21.21
11.273	31.449	16.741	16.144	14.535
12.675	12.411	19.926	13.272	16.75
15.384	23.452	13.072	17.148	16.892
11.598	11.599	21.018	15.413	25.477
15.131	19.938	15.967	21.173	22.877
22.597	21.048	13.265	15.412	14.512
13.526	15.292	16.876	26.019	22.537
15.695	13.856	12.379	26.315	10.637
24.726	13.642	19.548	26.568	13.621
19.148	22.613	15.002	15.952	16.084
12.106	13.98	16.487	18.538	19.144
29.705	16.953	12.112	27.756	12.637
26.674	15.957	13.723	15.654	20.977
20.976	18.821	11.375	21.355	20.156
37.089	32.84	19.098	17.861	15.291
20.961	22.173	16.439	21.98	22.605
30.141	12.472	14.487	22.925	21.151
18.58	21.534	18.543	20.197	19.959
22.002	12.186	23.885	23.087	21.509
21.25	18.447	20.394	15.458	18.405
20.9	68.975	26.045	18.662	10.409
			Mean	**19.238**
			SD	**6.496**
			SEM	**0.53**

Figure 5. 37h *in vitro*

Cell Pixel Intensity

12.637	12.444	22.902	9.16	8.684
10.462	10.005	19.218	6.186	14.092
7.022	10.816	21.701	8.715	23.304
7.569	11.35	22.903	9.564	14.142
7.77	7.325	21.721	8.855	14.078
10.208	8.614	19.121	14.813	10.122
8.666	5.711	15.729	8.309	18.185
10.636	6.587	25.581	16.629	11.851
11.605	7.691	22.63	17.568	17.599
12.19	8.721	25.53	11.539	25.53
11.116	9.856	18.033	15.795	12.886
11.818	22.97	18.23	16.59	23.792
6.415	12.316	14.321	10.578	10.547
6.872	10.432	19.758	10.192	15.306
8.293	10.292	20.183	10.347	23.008
9.485	7.065	20.487	11.96	22.855
5.273	7.292	18.265	14.47	21.085
6.642	12.909	16.272	26.415	16.909
5.919	8.238	17.129	8.6	16.015
11.092	23.876	15.625	18.389	11.179
5.726	17.813	25.257	21.647	18.486
6.138	26.437	16.002	21.902	8.449
15.251	17.455	13.744	16.205	17.655
9.659	17.94	10.931	22.338	21.877
14.015	25.675	13.707	19.402	17.183
9.462	12.724	11.925	14.274	14.319
8	16.426	12.835	20.441	16.645
19.212	28.767	10.402	22.333	17.524
14.689	43.295	12.284	34.98	15.285
5.523	28.231	9.809	18.894	18.208
			Mean	**14.898**
			SD	**6.434**
			SEM	**0.525**

Figure 5. 43h *in vitro*

Cell Pixel Intensity

20.029	13.786	35.078	20.805	36.262
24.018	18.262	19.778	21.498	15.339
15.049	12.917	18.374	18.344	16.933
15.036	14.577	19.681	25.62	16.507
22.102	21.918	15.933	27.024	20.11
27.777	20.078	15.153	21.491	22.657
20.372	25.633	9.528	25.599	24.092
21.293	14.798	17.507	17.257	23.149
18.117	23.824	19.705	26.711	14.905
20.604	20.33	9.113	18.183	13.558
37.159	18.981	17.651	17.371	30.452
23.232	22.56	11.487	16.877	13.874
21.924	31.086	15.708	20.958	25.058
16.143	23.838	12.566	13.981	19.33
22.706	14.762	16.767	15.43	24.123
25.962	8.298	18.304	22.829	17.117
25.861	23.118	15.606	12.312	25.363
19.028	15.382	18.664	16.587	25.543
19.043	20.713	20.049	17.451	20.237
16.218	19.401	18.714	18.722	18.071
15.509	19.727	11.928	25.079	18.647
18.014	27.686	35.893	15.571	20.785
13.845	17.093	13.929	9.155	20.848
15.084	21.571	14.572	13.522	27.491
12.897	23.31	17.753	14.927	20.901
25.767	15.559	20.188	18.712	16.66
18.654	7.335	21.441	10.44	24.565
18.017	11.052	11.496	11.199	27.397
14.775	13.256	21.423	24.414	25.382
16.557	12.783	15.82	17.533	16.598
			Mean	19.212
			SD	5.465
			SEM	0.446

Figure 6. 1h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
38.554	37.279	108.66	80.433
34.089	44.883	102.218	85.578
40.145	30.95	92.519	84.498
42.166	28.813	55.23	96.757
34.383	36.031	69.748	127.864
32.379	40.726	91.584	83.98
34.19	38.882	64.015	104.861
42.964	20.743	61.764	85.926
46.24	20.902	62.284	84.458
36.91	25.629	119.157	88.871
28.22	38.981	99.743	90.247
27.669	74.463	84.01	101.665
37.921	40.45	85.847	81.687
38.976	68.426	105.03	119.706
36.958	59.707	98.59	109.964
38.654	62.204	107.455	108.829
30.282	25.299	100.469	86.461
31.571	40.516	118.352	91.268
33.368	57.3	86.657	83.339
29.866	29.468	110.215	101.444
46.156	37.31	84.886	111.933
47.166	42.922	102.775	95.341
32.251	40.973	94.41	73.64
38.74	41.125	99.574	67.906
49.241	35.133	128.259	65.902
24.416	33.238	111.738	83.917
26.482	42.85	101.318	86.592
44.902	33.145	93.734	81.112
39.011	49.567	70.692	91.004
35.26	52.739	88.186	85.805
27.644	47.374	95.289	85.576
45.066	47.23	91.887	84.6
32.766	47.185	96.936	75.338
24.85	53.405	112.095	75.28

33.238	49.618	115.206	94.322
26.02	43.303	107.57	96.116
42.146	28.983	96.065	92.538
36.881	50.276	105.115	106.866
49.664	41.908	111.31	90.775
49.542	45.556	81.869	83.755
46.499	50.63	98.548	88.938
53.894	36.87	75.3	106.415
57.56	37.596	106.944	106.009
46.881	46.473	93.472	122.306
46.154	47.509	88.435	127.208
57.069	43.181	146.821	148.688
39.385	48.561	98.956	119.983
61.328	40.891	106.009	106.89
32.929	38.682	77.226	69.372
50.743	40.038	67.946	116.134
43.341	38.903	76.14	106.718
56.537	52.123	64.071	111.685
52.127	35.21	67.073	138.676
49.921	45.62	71.794	105.485
41.931	39.949	111.185	139.204
42.282	35.26	88.022	116.308
53.09	39.505	94.685	101.812
35.734	46.439	151.184	128.205
41.883	52.041	104.464	98.215
36.409	46.136	99.722	112.027
40.667	42.368	105.807	95.228
32.861	63.784	87.333	104.98
42.925	37.222	112.458	72.241
35.446	66.945	127.922	93.731
39.518	38.686	101.371	114.339
39.401	38.307	116.151	94.118
43.901	34.634	108.312	95.135
38.427	55.298	179.287	92.947
37.954	44.011	98.442	119.427
43.351	60.701	118.7	89.711
36.435	44.004	117.317	87.49
49.89	44.432	94.129	89.269
37.642	43.928	72.59	70.514
47.047	31.687	82.244	93.589

	35.296	30.445	85.39	75.986
Mean		**41.58**		**97.287**
SD		**9.504**		**19.321**
SEM		**0.776**		**1.578**

Figure 6. 7h *in vitro*

Cell Pixel Intensity

61.433	39.082	55.306	46.908	39.056
68.668	74.644	57.028	46.031	44.207
43.854	40.335	44.499	74.239	54.552
61.296	39.349	29.338	47.023	42.967
46.045	52.568	43.675	35.773	48.036
51.408	60.447	66.218	32.185	60.265
49.806	34.644	81.84	43.072	51.039
45.991	39.107	57.946	47.493	53.528
52.499	47.732	42.14	61.368	50.568
56.113	41.471	50.83	27.409	39.408
44.876	48.212	50.352	44.526	45.28
60.049	63.992	48.102	42.197	48.666
58.623	42.831	60.117	43.344	32.783
46.236	38.565	44.167	43.974	37.279
49.766	55.161	41.097	44.544	58.36
40.329	29.119	43.668	35.893	52.24
35.408	47.884	40.849	44.455	31.553
64.938	47.823	57.036	49.772	69.163
51.421	48.509	43.817	33.664	56.016
56.371	62.884	55.94	35.603	52.53
44.825	72.285	59.898	54.67	52.985
38.543	54.047	38.404	43.94	42.874
41.088	52.244	47.102	84.254	51.466
35.869	42.06	41.698	56.969	38.529
39.645	49.52	42.098	35.43	66.68
40.836	46.193	65.02	43.833	39.982
48.999	44.766	42.156	46.872	74.605
50.541	49.371	53.004	37.262	45.256
49.711	51.073	62.165	41.943	46.447
40.494	33.34	68.447	43.699	34.605
			Mean	**48.588**
			SD	**10.587**

SEM	**0.864**

Figure 6. 8h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
58.78	52.982	82.555	75.847
57.747	65.645	68.612	100.156
88.167	47.549	78.441	82.888
55.331	44.95	87.242	69.551
56.215	59.375	100.28	107.547
72.408	34.009	87.407	76.511
46.899	61.482	67.769	87.002
45.004	54.479	67.629	67.139
55.715	46.715	84.974	86.537
60.846	60.287	86.617	81.909
66.492	68.194	82.179	69.291
64.293	40.91	77.228	77.38
49.746	72.412	108.903	87.25
58.334	48.847	103.386	107.995
61.604	58.691	72.433	80.69
61.89	48.603	97.96	87.536
54.117	38.797	89.678	78.691
70.133	41.056	79.835	98.3
62.177	33.931	125.715	89.974
53.813	45.507	99.552	78.184
40.221	51.094	119.862	105.468
36.566	57.842	75.598	85.489
60.668	63.365	115.85	35.195
38.027	53.26	82.28	88.639
30.575	56.501	81.92	116.765
66.106	51.446	78.595	64.98
33.148	40.219	77.537	97.401
61.019	41.243	94.277	117.884
33.379	49.914	68.261	90.75
33.79	111.68	96.031	123.999
45.464	47.032	77.023	74.035
27.919	56.633	78.168	52.938
31.49	52.465	86.724	74.691

47.858	45.793	90.786	78.428
48.352	54.868	99.646	86.939
39.931	55.632	100.815	78.669
39.572	61.815	123.402	117.785
45.032	47.298	88.478	80.885
34.508	44.098	115.13	72.11
41.494	44.791	112.509	83.124
47.665	42.817	89.792	78.792
47.172	45.243	88.926	106.775
51.054	50.366	105.38	124.601
51.732	46.811	96.903	97.829
48.676	44.39	81.85	98.18
53.485	48.981	80.936	89.703
51.608	44.176	61.829	94.355
61.868	34.234	74.737	88.385
59.857	30.302	109.105	90.066
50.013	30.488	87.244	80.173
56.904	42.824	66.172	100.393
43.741	46.478	100.174	83.52
49.464	36.768	106.335	88.971
48.785	36.085	78.373	77.569
43.744	72	77.615	72.833
52.114	53.563	90.613	69.536
47.968	56.223	77.619	68.975
57.963	71.618	81.115	79.978
26.339	58.558	87.528	81.121
65.744	55.379	88.618	86.281
53.592	41.686	88.686	84.05
41.027	44.694	113.622	72.906
38.295	72.065	107.553	75.631
54.047	58.753	115.644	78.192
50.917	69.315	90.681	101.507
44.422	42.495	74.579	80.751
66.18	51.299	86.395	99.713
60.452	54.573	87.598	97.439
59.023	62.478	85.744	122.311
41.439	47.428	85.34	116.106
51.776	54.728	91.26	86.998
62.382	51.668	124.648	120.02
60.527	54.67	120.512	94.755

	55.932	49.193	86.518	87.383
	61.962	42.94	83.622	98.535
Mean		**51.396**		**89.17**
SD		**11.752**		**15.876**
SEM		**0.96**		**1.3**

Figure 6. 14h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
90.151	34.167	118.37	114.686
63	57.273	98.795	164.545
55.153	45.647	149.86	84.884
64.869	33.306	92.14	63.91
56.735	39.212	81.317	53.338
31.334	39.443	66.922	61.001
36.322	31.419	121.712	126.681
52.171	41.841	127.75	115.239
87.06	27.071	146.442	122.763
87.704	61.037	90.12	60.162
39.442	52.96	87.537	65.015
32.5	41.077	85.521	48.016
49.98	43.782	68.953	101.011
76.512	43.219	79.802	117.845
77.991	57.645	89.82	89.354
90.6	64.796	92.232	66.311
47.956	77.791	94.24	61.176
84.966	29.245	66.067	90.491
59.495	63.062	72.781	115.333
38.591	70.515	102.666	93.486
82.606	82.962	72.256	88.932
59.459	69.955	71.4	100.19
44.073	46.083	83.076	73.929
43.862	56.854	61.91	56.367
39.295	41.811	146.117	67.433
40.239	38.985	103.685	71.463
30.327	47.307	80.399	80.814
62.382	30.189	88.915	99.162
56.777	26.536	71.341	107.916
55.248	29.482	74.846	100.737
58.089	40.362	63.653	78.533

43.194	39.768	48.137	73.886
27.527	37.989	125.286	74.708
35.747	33.651	136.34	100.096
81.401	38.427	129.034	100.331
25.834	32.01	105.365	98.259
37.272	49.577	122.833	97.288
35.988	37.382	118.941	69.688
55.129	53.853	120.35	113.131
54.524	55.105	113.893	49.371
62.252	52.481	101.893	81.211
51.501	39.157	112.739	70.435
53.902	86.146	91.91	68.79
63.804	46.488	94.476	68.583
37.052	35.874	93.125	64.172
41.007	59.874	92.14	76.226
30.378	50.116	87.459	92.019
60.716	35.284	82.988	78.186
54.143	64.488	121.732	81.664
87.677	33.282	77.71	48.538
42.586	59.118	71.195	74.105
57.257	68.857	90.438	105.371
36.242	44.712	91.211	67.608
108.562	53.316	75.828	84.993
43.47	69.429	92.816	31.185
55.718	51.728	84.555	64.854
55.495	37.745	60.002	51.293
52.175	62.183	77.051	83.857
58.575	46.383	106.096	45.098
46.986	45.258	97.081	57.564
39.177	43.796	78.328	98.788
58.628	60.382	89.84	90.075
62.836	67.368	105.705	103.815
67.177	38.281	67.268	86.11
57.042	61.49	66.841	57.535
41.419	54.848	66.788	94.05
39.121	41.035	76.665	126.544
46.095	30.333	76.604	98.605
39.761	48.045	77.71	107.474
24.62	58.99	116.803	95.955
39.427	44.317	79.371	98.692

	38.379	43.177	94.815	76.836
	29.124	56.237	79.318	89.265
	25.674	74.375	84.033	93.515
	33.065	68.244	87.98	39.546
Mean		**50.721**		**87.943**
SD		**16.249**		**23.199**
SEM		**1.327**		**1.894**

Figure 6. 20h *in vitro*

Cell Pixel Intensity

34.569	51.661	30.072	46.036	30.054
45.641	51.736	41.599	82.505	44.913
36.044	51.55	38.722	52.458	52.125
41.329	53.548	40.839	49.53	31.196
33.809	64.353	39.863	26.15	39.684
29.378	42.051	46.316	25.331	47.071
47.055	36.735	39.307	50.591	35.919
37.25	47.269	37.513	28.034	40.285
63.764	45.388	31.981	30.381	41.981
39.211	39.213	53.132	29.89	36.275
33.242	40.373	64.118	37.597	42.86
38.668	37.19	58.749	27.309	41.054
35.231	48.03	32.733	37.036	38.287
49.045	40.042	40.17	44.403	56.945
39.602	50.273	44.373	38.238	51.185
58.998	55.115	43.46	43.357	33.35
54.703	56.414	42.248	49.669	37.912
46.207	44.635	52.873	49.889	34.834
50.105	43.491	35.697	44.383	51.668
37.785	52.882	50.569	41.429	27.478
51.386	45.217	76.869	42.425	31.932
25.501	48.516	39.607	41.59	29.755
52.804	47.425	77.582	36.555	37.277
47.019	40.168	65.234	39.015	37.006
43.07	41.643	54.464	46.424	41.184
55.113	47.031	36.428	35.097	39.151
59.196	37.114	37.6	41.221	35.417
47.086	46.825	32.441	34.26	42.687
82.216	28.684	37.174	46.95	30.202

64.153	36.831	42.748	43.248	43.97
			Mean	**43.598**
			SD	**10.502**
			SEM	**0.858**

Figure 6. 26h *in vitro*

Cell Pixel Intensity

45.361	33.967	9.479	30.649	23.821
32.009	26.199	33.268	34.851	20.323
48.498	30.357	37.803	42.524	16.04
26.918	13.7	22.605	30.811	20.001
44.235	27.625	35.568	36.796	7.908
35.864	31.449	36.94	29.174	15.405
29.24	25.529	27.254	38.347	24.623
30.246	18.197	27.092	47.905	28.617
31.394	39.405	31.653	44.883	11.376
35.374	22.513	67.388	21.331	32.223
27.529	31.117	25.502	31.985	18.923
16.269	39.535	41.626	26.389	30.426
22.197	34.111	22.312	32.698	18.885
10.062	28.748	39.736	48.281	21.947
18.472	30.469	36.929	22.629	21.782
27.33	43.385	36.843	34.575	16.89
22.245	22.347	32.877	45.53	22.062
22.021	20.674	35.196	37.253	24.541
16.799	21.467	29.638	45.382	19.452
30.955	24.527	44.878	32.033	32.892
11.239	23.057	24.655	28.721	29.808
23.855	16.592	22.658	24.079	38.453
26.481	20.015	11.377	31.25	33.392
14.372	32.053	31.026	43.016	29.01
26.772	30.46	23.448	39.661	19.406
7.444	14.714	20.553	54.733	27.633
17.581	24.384	29.105	41.75	28.445
18.131	20.56	21.21	39.195	27.583
27.533	20.792	17.836	39.805	26.466
16.335	25.379	21.697	14.788	27.174
			Mean	**28.272**
			SD	**9.868**
			SEM	**0.806**

Figure 6. 32h *in vitro*

Cell Pixel Intensity

46.918	38.191	46.475	28.836	9.318
39.378	23.79	30.262	39.561	8.486
29.754	21.475	42.472	33.633	32.118
35.735	24.718	29.59	41.172	31.8
22.812	37.881	40.714	41.153	39.404
11.016	32.82	34.511	30.103	47.352
22.834	24.103	38.454	40.902	10.83
34.98	29.98	22.312	38.408	11.132
26.224	35.089	36.046	47.301	15.836
31.712	28.37	42.895	36.036	35.616
40.918	34.736	31.756	68.318	24.556
37.555	28.786	28.062	21.507	21.388
38.485	37.288	41.034	57.769	21.842
35.449	33.313	26.234	26.785	45.078
40.219	36.657	41.322	64.568	58.49
39.618	37.384	32.081	39.327	35.241
28.581	29.5	73.459	41.155	34.398
28.696	44.215	34.631	41.636	46.309
54.758	33.318	28.669	45.082	27.989
56.909	49.084	36.178	39.485	32.197
32.095	37.457	42.85	25.744	37.69
49.768	18.373	35.026	17.79	24.641
45.469	34.086	35.911	10.004	25.783
28.489	18.143	30.834	9.887	38.262
47.304	57.55	30.71	13.48	14.153
45.873	35.059	32.986	34.728	14.854
30.969	58.85	31.757	39.285	45.111
27.036	42.846	32.708	28.34	34.22
29.547	48.524	23.612	10.527	51.875
28.178	31.961	32.998	10.96	46.073
			Mean	**34.153**
			SD	**11.847**
			SEM	**0.967**

Figure 6. 38h *in vitro*

Cell Pixel Intensity

27.564	27.999	13.779	36.949	26.235
20.797	14.601	21.42	29.962	46.498
16.388	8.961	24.525	23.85	26.77
14.114	10.278	18.299	23.125	19.759
27.236	13.555	28.293	58.412	44.633
33.126	21.15	29.431	26.588	30.668
13.814	10.09	30.491	14.091	12.557
22.751	27.029	22.965	14.81	12.663
22.459	8.465	24.173	20.107	21.922
24.178	10.147	22.366	24.098	21.631
23.279	34.268	26.838	28.962	30.517
15.594	45.638	55.369	41.924	34.341
28.709	34.721	26.205	20.753	24.86
65.375	16.048	19.125	22.274	17.655
20.863	27.337	24.665	41.929	31.789
38.838	29.518	18.977	24.92	27.869
41.437	23.31	20.084	36.533	24.044
21.374	19.489	36.889	27.474	19.662
18.056	30.312	23.837	17.196	31.592
18.226	29.306	18.837	24.771	23.829
34.139	26.834	22.174	13.307	23.669
23.951	21.091	15.513	14.785	18.881
22.861	28.506	13.494	32.103	24.759
22.53	20.331	20.667	28.879	26.403
20.772	25.29	19.556	22.253	16.776
36.078	19.126	27.06	28.808	26.419
25.388	22.519	22.369	11.691	21.92
32.091	17.088	19.239	18.108	24.113
26.392	26.376	41.28	14.815	13.739
29.611	30.802	19.489	8.264	13.444
			Mean	**24.58**
			SD	**9.254**
			SEM	**0.756**

Figure 7. 1h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
46.503	60.688	97.379	132.135
40.14	58.38	105.215	145.401
42.154	44.261	95.058	81.248
32.923	69.53	123.438	118.049
34.335	59.117	106.239	93.916
47.311	44.192	107.041	104.052
34.045	73.067	116.111	126.5
51.634	39.645	70.853	116.051
88.305	24.142	97.913	95.452
51.038	33.411	70.16	109.106
27.819	28.763	78.416	105.583
53.71	42.374	82.41	116.324
48.779	40.606	98.316	100.436
48.008	31.554	102.817	130.428
76.995	27.378	106.927	113.135
68.505	34.887	67.262	74.244
56.382	44.986	88.434	66.036
24.568	59.107	78.235	90.636
30.636	79.831	124.547	96.524
73.438	84.19	135.208	100.989
36.125	60.894	109.062	94.565
44.18	59.499	87.684	92.364
73.005	56.186	97.064	63.466
51.641	27.714	134.527	117.5
45.217	28.582	106.329	133.227
74.992	34.94	133.736	112.99
73.694	44.716	97.433	148.292
46.057	62.034	96.091	147.455
57.234	63.642	82.898	170.816
63.918	47.557	87.712	132.136
48.327	28.425	95.108	92.685
60.184	22.082	107.85	146.687
67.094	45.916	131.261	131.723

53.388	57.216	102.27	121.985
53.098	70.096	80.745	127.359
74.395	48.581	100.294	108.584
46.877	29.778	74.461	173.333
95.781	44.6	96.429	137.161
56.39	57.332	89.273	166.645
69.531	46.524	118.879	115.084
54.054	35.805	121.593	133.921
56.157	26.418	101.247	153.013
58.765	36.094	137.813	103.352
95.992	72.05	100.2	109.729
31.663	61.162	147.828	131.238
28.354	47.541	113.521	115.137
37.695	35.538	133.844	93.005
32.895	32.095	102.003	99.455
33.355	42.983	124.498	66.023
36.58	51.022	116.525	82.578
37.673	37.436	91.606	87.849
46.405	64.26	126.796	96.584
37.891	49.693	117.648	93.392
57.471	40.422	102.527	90.043
73.201	52.418	146.51	74.143
41.112	63.453	120.123	92.915
29.071	66.047	129.491	94.561
51.312	49.341	126.727	130.949
44.571	48.784	146.635	101.571
67.122	21.269	122.968	114.129
48.892	115.634	125.085	122.29
72.023	69.858	111.781	113.035
48.161	36.285	103.661	103.972
46.777	32.865	98.604	144.108
58.6	23.486	132.634	124.679
40.284	34.453	98.059	116.269
31.751	38.051	174.775	109.599
59.939	38.992	134.63	132.484
53.462	41.675	129.19	97.284
43.911	51.637	121.8	88.317
36.723	47.35	184.81	114.34
56.67	79.784	127.281	97.606
46.44	55.161	153.263	108.877

	46.663	38.16	92.096	156.959
	40.116	76.455	96.552	109.57
Mean		**49.868**		**111.658**
SD		**16.559**		**23.561**
SEM		**1.352**		**1.924**

Figure 7. 7h *in vitro*

Cell Pixel Intensity

47.361	40.636	28.339	38.319	29.629
33.624	36.777	28.151	45.56	35.232
33.737	45.818	50.245	48.359	23.904
30.25	30.743	39.257	38.818	36.641
26.225	39.585	42.961	42.896	36.859
50.762	45.502	52.19	44.932	50.53
28.06	54.981	46.333	48.237	37.937
46.168	36.245	35.788	45.047	25.543
53.459	37.061	41.014	45.511	30.351
30.103	31.366	44.573	43.494	22.181
36.311	40.529	27.085	62.641	28.749
33.589	42.785	32.172	31.444	29.18
25.57	55.554	43.656	48.465	45.812
44.048	38.868	33.118	41.494	21.612
42.044	51.745	41.985	43.617	43.86
36.412	50.668	61.805	49.646	24.694
38.885	41.887	32.408	47.867	37.974
53.196	34.312	28.667	52.971	32.029
30.89	16.876	38.94	36.833	44.688
38.225	25.575	42.674	45.154	62.164
40.267	27.501	40.865	37.465	46.556
18.469	38.678	59.521	33.263	45.325
31.871	26.79	47.932	33.819	44.931
34.125	32.536	41.164	51.246	34.813
29.899	26.285	46.432	50.676	41.96
42.384	45.304	59.424	31.437	57.119
40.675	50.763	48.376	48.67	37.626
56.534	28.561	37.302	39.203	39.099
55.471	34.94	37.603	57.899	47.468
54.617	29.982	46.062	37.66	30.599
			Mean	**40.039**

SD	**9.53**
SEM	**0.778**

Figure 7. 8h *in vitro*

Cell Pixel Intensity

23.695	31.482	23.24	29.854	20.669
22.768	20.632	17.88	30.201	15.9
33.694	19.778	32.214	43.116	17.77
13.627	26.341	20.544	17.51	19.719
33.954	30.559	25.675	26.362	21.489
24.201	23.73	20.663	21.454	25.794
27.096	22.572	26.89	26.842	32.852
19.383	42.207	34.739	25.384	25.124
21.642	16.595	19.984	27.262	27.31
54.142	18.61	27.113	18.836	21.777
41.734	24.99	26.864	21.146	20.534
21.033	23.705	31.375	28.392	23.341
16.993	31.215	15.707	25.287	26.055
25.585	28.439	21.617	28.956	20.779
11.84	28.12	24.339	49.118	23.605
25.764	76.557	9.325	31.446	18.765
21.578	35.272	30.682	29.427	25.133
37.116	20.259	31.048	28.434	23.345
43.257	13.814	26.594	54.347	24.36
42.924	16.848	23.082	29.436	23.666
17.404	42.305	23.586	29.682	21.934
16.859	18.013	18.752	30.681	15.173
31.811	10.042	23.391	30.62	18.503
19.205	14.914	16.857	13.512	29.465
21.055	32.463	27.221	26.423	17.95
26.32	14.96	19.241	33.983	25.093
21.455	19.449	25.525	25.838	31.532
22.606	23.943	16.333	24.964	27.287
26.995	21.237	23.083	24.636	23.197
25.952	25.542	36.479	31.298	27.011
			Mean	**25.639**
			SD	**8.714**
			SEM	**0.712**

Figure 7. 14h *in vitro*

Cell Pixel Intensity

29.966	44.308	33.358	27.805	34.183
51.644	31.769	51.625	36.121	33.592
33.542	22.915	39.824	36.913	30.964
30.745	50.383	43.924	39.202	39.158
35.94	42.249	48.09	34.756	51.18
38.842	37.169	43.981	30.737	68.66
25.092	37.24	63.224	34.438	40.693
27.164	31.034	42.312	25.87	49.67
24.028	38.271	34.201	27.39	37.82
26.185	54.199	37.83	31.873	34.357
22.798	28.435	59.2	33.892	49.606
26.21	36.107	56.693	39.709	49.187
33.018	36.836	34.931	33.079	48.823
31.646	36.89	42.351	22.719	34.215
41.822	41.941	41.14	37.144	44.786
39.465	29.321	48.313	27.567	37.346
29.449	33.085	44.35	34.026	30.373
38.663	44.892	22.313	27.127	30.572
43.649	41.288	26.48	32.845	34.307
39.035	36.296	23.136	32.319	32.881
21.489	27.24	35.704	32.017	35.697
35.601	41.192	38.568	34.722	34.455
39.799	36.348	31.822	53.533	35.084
43.531	30.681	31.916	35.365	41.283
56.982	30.479	28.162	37.078	48.074
48.621	39.875	39.388	42.442	31.673
37.695	29.874	44.962	46.162	28.997
29.299	33.857	28.065	36.045	31.09
50.518	54	36.123	30.828	38.464
37.062	26.987	29.5	39.204	25.936
			Mean	**37.015**
			SD	**8.625**
			SEM	**0.704**

Figure 7. 20h *in vitro*

Cell Pixel Intensity

21.69	24.11	21.716	27.645	33.326
22.727	25.251	21.276	40.223	29.306
30.236	27.716	25.19	28.307	22.493
35.9	32.503	29.023	32.787	31.667
26.651	29.919	26.32	25.577	27.366
29.467	21.005	30.602	24.686	36.489
32.527	25.159	33.689	25.683	39.53
21.939	27.919	29.664	22.961	39.55
25.498	25.967	29.287	23.026	37.468
31.037	23.904	36.747	27.178	48.907
27.294	22.748	30.832	35.127	30.928
26.649	29.197	26.759	27.693	26.869
34.172	28.711	34.66	24.909	29.766
30.921	28.383	25.237	23.726	27.01
28.134	30.917	29.168	25.822	32.948
37.255	30.696	25.611	19.245	20.595
29.296	24.429	21.377	22.079	19.627
21.984	24.053	21.793	22.964	21.9
33.705	37.852	22.786	21.76	53.173
32.881	35.784	25.164	27.715	34.046
40.739	34.8	28.125	20.836	30.376
37.393	31.205	19.995	26.61	38.22
27.229	35.243	25.632	30.068	30.903
37.568	38.01	27.433	23.592	32.577
23.142	27.855	29.976	33.321	30.578
21.896	24.131	26.514	31.445	27.901
21.212	21.381	40.017	24.432	27.201
25.46	22.402	37.263	37.401	46.718
21.427	22.14	27.578	25.86	23.621
23.962	23.857	53.803	26.044	37
			Mean	**28.915**
			SD	**6.373**
			SEM	**0.52**

Figure 7. 26h *in vitro*

Cell Pixel Intensity

18.619	18.304	27.96	18.611	22.692
18.209	24.441	23.746	21.328	14.012
17.694	20.221	14.799	20.139	18.109
12.346	16.373	16.567	17.1	11.075
18.836	23.875	19.644	14.948	14.676
18.985	13.847	25.565	15.269	21.336
15.039	24.802	24.483	29.015	23.237
21.333	21.723	14.5	18.246	18.097
13.613	13.186	19.301	25.485	18.781
22.489	16.718	23.942	27.987	15.577
9.638	9.51	23.106	9.389	12.148
18.984	16.005	24.031	25.991	25.856
16.357	13.72	21.632	14.375	13.197
18.681	19.449	16.055	16.83	16.672
17.823	16.643	17.426	26.408	13.493
21.85	17.216	23.858	15.626	12.333
17.777	11.785	11.421	22.896	14.479
12.102	15.709	26.054	16.683	13.431
22.561	17.952	9.575	15.769	24.966
17.13	17.29	18.428	16.525	25.026
7.376	29.993	18.648	20.293	19.595
15.646	22.291	17.369	20.386	13.785
18.784	13.581	18.224	20.493	18.293
18.138	18.144	10.734	19.26	16.643
8.817	8.071	22.839	12.537	26.836
27.775	13.772	14.568	11.538	11.923
16.349	17.211	16.692	19.735	20.821
22.171	12.733	15.822	16.382	31.03
19.745	19.334	20.33	16.62	21.951
15.684	16.276	21.907	21.399	22.219
			Mean	**18.263**
			SD	**4.776**
			SEM	**0.39**

Figure 7. 32h *in vitro*

Cell Pixel Intensity

16.689	15.68	21.011	14.47	14.61
15.174	15.55	11.001	7.138	15.015
13.962	18.196	13.385	13.724	13.282
14.191	16.222	18.2	12.229	15.532
19.111	26.309	12.615	13.302	13.961
10.086	15.072	12.24	14.381	14.342
26.689	16.917	10.264	18.775	13.531
15.149	12.972	4.279	24.184	15.016
11.558	20.612	20.11	24.375	14.966
15.916	12.421	16.151	12.803	15.762
13.274	10.846	11.235	14.559	18.346
10.356	11.298	12.524	14.241	14.494
23.169	9.588	16.525	15.483	9.184
11.897	11.002	10.695	17.296	20.203
14.123	24.872	15.807	15.93	17.336
20.669	7.623	6.627	12.453	9.745
15.761	11.171	8.675	11.097	10.188
19.39	15.011	9.105	17.09	5.842
17.48	17.49	16.9	14.321	15.631
13.82	20.487	16.912	8.682	15.653
16.21	10.351	20.525	16.154	15.954
14.937	12.631	20.434	11.004	27.221
12.614	12.485	19.516	10.979	12.095
11.586	21.767	10.734	20.223	12.526
34.542	27.268	10.967	21.188	13.397
7.835	9.389	13.53	13.705	18.406
15.335	12.255	16.747	13.451	21.919
7.786	9.249	15.533	10.788	25.082
10.992	13.93	13.825	12.438	23.399
17.857	16.48	11.936	11.267	9.467
			Mean	**14.941**
			SD	**4.745**
			SEM	**0.387**

Figure 7. 38h *in vitro*

Cell Pixel Intensity

24.875	21.45	19.397	15.092	8.284
21.296	13.911	25.437	26.211	12.05
28.093	11.598	23.687	32.473	13.619
9.649	19.447	28.799	15.182	18.056
13.705	17.874	20.009	10.257	13.959
16.607	15.932	33.432	11.249	15.685
12.45	15.171	17.105	7.851	6.457
15.161	16.708	11.788	7.327	15.341
15.236	12.341	18.081	10.798	25.217
18.145	16.842	8.836	15.378	17.941
13.315	9.134	9.424	23.694	13.495
9.141	5.832	17.488	20.494	24.851
11.478	9.773	11.045	13.313	15.293
30.832	10.507	12.517	10.188	24.244
22.191	34.076	11.176	33.75	8.253
13.455	15.298	11.615	22.857	18.61
13.926	17.151	12.966	15.829	11.435
10.126	13.935	10.987	24.795	10.24
10.894	19.733	28.491	21.904	11.355
12.813	19.935	26.733	19.209	23.949
10.856	31.394	17.979	20.105	18.051
6.014	55.076	22.205	22.858	18.23
7.021	15.864	30.431	18.327	15.762
8.394	10.567	18.164	21.813	29.092
12.434	12.378	7.643	16.948	14.964
13.154	10.776	9.897	12.464	9.646
9.423	7.985	11.846	7.387	10.827
17.406	24.393	18.332	6.6	7.667
8.22	22.066	22.444	7.79	9.789
17.178	13.5	11.917	12.778	11.617
			Mean	**16.286**
			SD	**7.304**
			SEM	**0.596**

Figure 8. 1h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
44.369	47.014	119.206	130.176
39.233	45.827	77.549	107.926
37.625	46.631	93.914	141.122
28.531	59.389	95.254	117.057
28.756	32.324	121.587	81.127
26.841	35.485	102.313	129.675
23.601	65.337	92.742	102.306
24.499	44.515	118.957	131.664
37.11	58.358	107.285	87.064
50.623	47.246	105.397	113.764
60.412	50.523	104.737	77.639
38.828	32.365	114.945	112.362
21.175	70.563	58.547	103.727
23.643	37.206	92.806	119.034
23.946	43.508	86.555	136.412
39.161	41.357	94.912	148.92
35.746	52.565	82.582	127.817
42.142	37.346	103.935	177.115
39.676	51.916	98.222	122.381
43.953	41.819	68.254	104.874
32.12	61.522	100.292	102.616
107.128	43.302	90.365	81.582
41.543	63.078	105.129	88.554
38.979	78.978	101.29	125.501
52.86	53.558	74.766	119.564
43.666	44.906	97.462	88.451
51.616	65.019	75.916	133.537
49.5	57.727	92.566	125.471
43.225	34.883	115.608	124.687
41.54	50.421	87.866	99.932
36.245	70.406	108.373	149.736
28.616	37.199	105.298	107.84

17.351	26.688	111.552	79.122
30.145	32.198	126.743	135.198
31.463	38.953	101.808	123.524
34.623	47.911	126.268	133.865
28.547	58.675	99.954	116.877
40.598	50.562	110.536	82.306
39.585	36.564	97.335	112.805
44.122	33.349	107.174	106.834
29.363	39.403	107.146	93.004
41.421	39.524	134.074	107.7
32.348	33.909	113.393	115.036
56.639	48.794	114.099	116.349
36.001	41.811	104.042	91.952
52.679	47.475	127.593	110.532
43.024	36.349	100.622	121.747
35.372	31.841	135.022	93.367
35.951	46.48	62.18	94.802
44.277	32.422	108.987	95.386
51.015	54.728	84.947	75.888
42.586	51.928	97.997	96.239
26.04	51.928	93.015	82.7
35.618	27.591	73.918	90.071
52.812	44.209	98.82	105.82
58.545	41.022	99.249	126.286
50.379	42.63	98.519	84.012
44.294	51.682	108.908	106.883
42.085	28.677	101.308	113.95
67.493	45.418	107.48	127.004
58.397	46.506	119.768	102.76
52.981	48.516	133.991	114.569
50.432	46.674	93.373	113.811
54.758	59.783	128.147	95.261
54.245	49.493	129.404	129.094
42.017	41.05	99.211	70.987
39.337	43.348	95.051	102.644
38.291	49.117	104.77	69.801
47.213	57.787	121.145	120.991
33.375	51.644	139.658	149.058
47.01	48.506	81.574	110.844
31.079	42.882	119.304	107.459

	26.162	31.444	119.578	90.275
	44.587	45.931	95.788	109.964
	57.937	43.802	125.407	106.071
Mean		**43.804**		**106.706**
SD		**12.103**		**19.118**
SEM		**0.988**		**1.561**

Figure 8. 7h *in vitro*

Cell Pixel Intensity

44.107	53.707	28.539	64.644	39.162
82.81	44.883	48.183	83.727	122.578
53.441	55.656	63.007	57.661	54.046
41.038	57.066	42.657	69.276	38.627
34.112	43.302	38.538	43.127	54.65
44.842	66.604	41.071	37.106	43.671
56.269	50.303	31.555	40.014	31.599
27.715	40.571	46.501	27.135	40.342
51.146	40.183	9.512	37.7	26.924
37.379	31.208	45.959	57.628	47.787
53.181	31.933	46.778	54.197	20.07
34.131	53.241	70.659	84.665	38.05
56.706	48.355	68.437	63.896	51.839
49.539	83.453	117.296	38.798	47.908
50.67	53.34	81.129	67.879	37.734
34.95	71.427	129.818	34.427	39.574
75.586	61.944	63.906	55.251	55.111
49.311	30.097	64.412	45.214	65.212
53.29	46.745	59.898	41.001	34.086
32.065	47.514	50.963	46.712	39.302
32.938	35.316	38.414	43.473	61.281
47.555	53.276	44.839	36.617	55.584
51.905	73.368	17.828	67.81	77.595
32.198	42.183	23.12	60.18	46.608
41.16	29.3	92.788	68.831	59.909
36.862	31.641	59.349	43.126	43.495
53.157	51.528	86.28	54.305	43.55
38.426	53.417	63.511	29.171	42.63
42.585	47.507	66.478	61.085	73.47
44.6	39.117	91.795	42.032	50.455

Mean	**50.831**
SD	**18.367**
SEM	**1.5**

Figure 8. 8h *in vitro*

Cell Pixel Intensity

40.357	36.759	32.208	44.952	26.654
36.444	38.129	37.939	32.423	46.516
33.828	49.538	35.23	38.719	38.76
38.766	37.169	51.569	40.489	34.784
45.405	43.777	34.835	40.787	34.719
41.476	44.571	60.44	36.025	35.882
38.176	47.653	48.113	35.794	25.277
43.066	44.833	50.269	48.741	33.178
38.365	43.039	42.444	48.452	31.09
41.748	40.855	48.808	52.776	35.573
40.426	50.562	45.43	41.831	35.942
47.237	44.955	50.839	41.429	41.713
43.078	37.988	57.56	37.261	35.002
62.349	36.781	40.346	35.379	37.006
44.957	46.819	42.571	35.484	41.084
47.975	51.869	39.119	29.091	40.326
38.037	37.692	33.853	54.35	37.615
41.228	38.51	26.661	31.2	38.392
26.894	46.283	44.6	42.08	35.462
30.332	27.518	34.389	36.466	36.016
32.785	29.559	39.153	46.573	46.707
37.592	37.712	36.422	40.404	36.392
48.582	36.658	40.471	29.796	41.199
49.208	41.05	42.524	40.17	36.737
46.805	45.728	37.803	36.973	32.08
37.233	37.169	39.138	39.83	36.278
43.804	41.058	45.825	36.745	35.355
44.518	41.536	43.049	47.774	33.229
46.341	41.382	36.463	33.028	33.182
36.104	48.253	43.839	37.867	34.64
			Mean	**40.201**
			SD	**6.541**

			SEM	**0.534**

Figure 8. 14h *in vitro*

Cell Pixel Intensity

23.464	28.387	14.301	32.335	13.569
31.949	15.064	21.917	22.737	37.152
23.927	19.817	24.423	16.345	36.643
23.48	19.96	41.792	22.606	28.148
36.561	20.036	17.586	16.104	15.245
27.114	19.176	26.508	20.934	14.097
26.583	20.307	24.143	20.058	18.211
15.112	13.728	18.645	17.964	18.291
18.975	19.419	21.807	12.441	18.337
25.968	8.444	38.62	17.341	22.345
15.268	18.704	31.157	30.638	12.815
30.699	26.104	22.154	12.077	9.642
26.709	20.819	20.851	23.687	20.913
19.65	23.846	22.367	22.057	16.687
29.81	16.207	13.456	25.956	23.589
49.908	24.392	23.004	22.997	15.823
15.797	19.863	20.607	30.195	14.351
31.029	22.129	24.996	17.781	24.28
26.28	17.636	37.866	18.156	20.005
22.363	16.55	22.792	34.218	14.629
16.771	20.182	25.016	10.889	23.181
25.465	15.419	22.532	26.68	19.042
18.334	24.246	20.897	16.746	22.717
22.655	20.198	32.851	14.586	23.665
18.457	19.154	20.326	29.537	24.076
16.742	23.958	13.844	10.3	24.749
14.504	17.894	28.61	32.651	26.36
12.557	33.033	20.515	18.077	19.634
15.914	25.241	27.067	24.699	11.164
22.604	22.259	21.394	21.201	17.737
			Mean	**21.92**
			SD	**6.686**

SEM	**0.546**

Figure 8. 20h *in vitro*

Cell Pixel Intensity

11.777	21.658	12.036	18.673	14.044
15.383	18.991	10.726	18.747	15.717
23.692	17.608	17.127	18.969	27.032
31.423	13.64	11.363	20.328	23.451
22.468	15.338	11.868	16.031	11.483
16.607	13.69	18.085	17.188	18.096
15.108	20.042	15.716	27.679	14.44
23.664	19.024	23.597	13.163	14.522
35.624	22.607	13.33	14.704	16.452
41.354	10.895	20.836	17.402	17.337
22.223	10.722	15.196	20.191	9.851
32.296	26.148	9.752	7.948	12.311
23.152	13.427	15.879	7.453	12.192
27.469	19.497	8.769	13.583	13.286
18.366	9.701	12.657	14.843	14.35
10.797	22.901	10.461	19.317	21.964
4.967	22.819	15.922	10.424	13.742
16.72	8.003	9.421	11.054	25.674
22.742	16.764	13.634	12.887	6.519
17.035	27.256	11.516	14.236	19.22
22.04	13.222	12.337	15.095	20.341
19.831	23.782	7.956	11.655	15.971
17.847	13.135	7.283	9.451	17.417
14.966	26.434	13.148	21.188	11.162
10.929	13.375	13.263	9.913	17.146
16.935	27.01	11.05	6.811	26.634
27.941	21.767	13.584	14.165	13.405
29.147	15.811	12.707	25.603	16.275
12.795	9.065	7.681	15.404	15.759
15.575	15.543	24.798	22.359	17.067
			Mean	**16.745**
			SD	**6.177**

SEM	**0.504**

Figure 8. 26h *in vitro*

Cell Pixel Intensity

30.266	37.146	22.87	9.787	29.697
53	29.886	38.208	12.077	20.553
18.577	16.902	66.898	14.356	14.477
16.274	31.01	38.016	10.654	9.221
16.436	33.119	43.337	14.915	15.105
17.391	17.097	32.781	25.896	25.724
19.553	53.753	31.968	27.084	30.452
23.554	41.295	30.703	21.376	16.173
21.369	33.273	40.236	20.903	35.25
33.367	23.478	41.448	28.919	32.029
21.337	10.385	17.111	30.249	26.366
21.211	21.119	23.396	21.863	31.542
36.943	15.61	21.263	31.815	18.768
27.667	29.094	29.549	14.038	39.662
20.723	43.68	29.499	22.111	11.128
33.007	23.476	25.999	25.217	42.411
56.652	21.334	28.18	25.56	36.186
44.125	42.38	24.884	32.382	39.41
19.076	40.358	32.62	47.071	26.586
23.069	39.189	22.747	21.469	24.273
23.519	41.946	22.854	33.192	39.674
27.825	27.444	21.04	26.719	21.247
43.202	21.572	28.074	44.116	25.168
37.792	32.031	23.501	41.402	30.895
33.733	29.333	35.263	31.529	29.819
35.604	20.081	20.692	25.029	45.38
38.453	33.725	28.54	33.228	34.767
33.837	36.605	19.265	20.91	48.588
29.64	34.503	15.098	26.155	19.426
19.156	17.844	10.719	32.397	30.618
			Mean	**28.432**
			SD	**10.183**

SEM	**0.831**

Figure 8. 32h *in vitro*

Cell Pixel Intensity

32.354	28.104	32.708	24.774	31.573
3.986	36.01	25.878	20.754	20.77
21.192	47.65	16.214	15.572	16.4
10.197	41.45	31.471	10.186	27.048
20.062	44.065	29.836	7.873	14.727
18.668	24.387	38.353	13.445	16.559
18.153	47.136	19.817	19.428	9.892
21.109	15.912	27.402	12.129	6.867
28.939	39.008	34.852	14.109	38.298
30.926	19.3	27.106	25.489	24.18
31.816	26.116	34.92	13.995	22.916
15.364	44.246	28.103	11.355	28.687
15.947	21.45	26.46	11.824	29.803
4.145	29.537	16.886	13.385	25.491
17.318	25.395	30.919	12.35	26.05
27.387	17.022	24.736	22.981	22.845
24.378	29.792	34.765	21.303	40.409
34.093	44.112	38.608	11.784	30.05
34.189	38.299	30.583	13.243	24.825
43.284	40.67	19.131	16.946	29.844
30.63	29.127	25.08	6.513	27.314
22.141	33.236	22.011	6.759	35.844
28.691	32.613	44.895	22.555	34.042
34.605	30.403	24.941	24.895	36.54
23.777	30.199	21.95	11.45	31.015
34.742	34.263	21.406	30.858	28.054
42.386	25.465	19.49	24.998	17.476
49.403	22.656	29.816	18.849	34.234
40.048	18.228	34.651	28.361	20.253
36.891	21.749	17.976	22.459	31.584
			Mean	**25.73**
			SD	**9.705**

			SEM	**0.792**

Figure 8. 38h *in vitro*

Cell Pixel Intensity

21.934	19.158	18.95	9.034	31.214
9.278	13.197	20.255	11.369	13.539
12.618	14.545	7.531	5.853	10.985
7.405	14.351	9.433	6.126	11.644
7.421	11.336	7.503	7.222	16.15
10.171	14.174	7.806	6.802	18.844
6.752	24.362	16.247	15.459	11.906
8.598	21.067	8.146	11.946	13.054
8.276	23.838	13.833	10.424	17.466
17.492	18.25	9.557	8.394	12.213
7.3	5.71	10.927	9.751	14.047
18.538	24.839	18.034	12.001	9.844
8.477	14.832	17.192	13.893	13.333
13.39	20.433	9.267	13.72	11.303
22.791	12	15.282	14.263	11.83
13.836	17.455	19.905	13.718	15.1
7.3	12.903	22.557	12.551	11.001
9.466	14.695	15.277	11.808	6.403
12.471	22.461	11.421	17.713	6.23
10.73	6.428	7.132	20.585	10.813
18.761	18.633	15.368	16.052	14.725
5.57	20.173	12.652	15.691	9.311
4.748	11.548	8.32	11.848	15.351
3.995	19.387	11.867	12.734	14.356
3.511	12.031	12.347	11.003	17.237
8.137	14.12	19.784	21.584	11.007
11.763	18.45	12.903	19.191	9.807
15.153	25.233	11.272	19.337	5.551
11.857	18.494	15.1	19.351	11.901
21.088	12.408	13.457	14.146	10.862
			Mean	**13.435**
			SD	**5.026**

SEM	**0.41**

Figure 9. 20H + Low Brain Fraction. 1h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
54.664	37.855	68.6	88.102
35.9	43.541	66.25	112.618
48.984	47.746	82.186	98.12
60.29	34.089	80.154	99.705
41.984	37.012	71.275	94.992
34.678	43.996	55.151	97.754
43.342	32.665	104.349	95.539
96.243	20.639	58.913	96.612
62.381	42.507	64.124	95.761
69.873	22.242	77.735	85.247
35.046	54.459	98.872	81.938
59.061	60.541	102.998	99.64
40.915	39.106	57.338	88.373
46.158	58.372	98.75	112.801
41.461	64.367	70.191	88.174
43.439	39.201	43.261	107.529
44.422	38.256	56.516	88.147
75.546	24.897	60.488	91.223
60.837	28.576	91.868	104.335
42.814	36.019	87.522	68.035
54.878	22.014	78.313	80.424
51.052	21.398	83.097	86.092
39.354	39.117	62.92	88.083
44.901	24.397	53.499	57.242
33.285	38.343	61.165	79.359
46.912	30.171	93.623	91.611
39.29	31.107	79.023	62.799
20.858	23.411	99.796	93.405
38.619	68.861	63.081	61.25
37.709	40.356	80.969	102.63
29.397	39.317	94.906	64.913

36.455	49.362	88.856	71.155
48.278	49.982	96.605	75.367
38.345	44.068	63.267	72.396
30.643	61.98	68.552	98.867
30.951	56.919	78.726	96.679
32.592	62.096	140.83	102.076
34.655	68.737	65.96	132.464
37.591	93.624	82.333	117.886
49.805	40.333	35.099	116.279
29.021	27.248	35.87	69.638
38.304	30.033	38.805	144.903
35.98	43.932	38.286	150.977
48.149	44.568	30.96	96.25
43.101	37.218	42.161	62.905
48.025	51.354	85.827	82.112
49.948	53.167	72.143	104.793
34.377	58.631	72.881	82.915
30.706	49.805	88.728	68.467
40.25	51.501	96.614	78.482
36.359	22.719	76.975	71.801
21.569	28.858	85.619	70.132
25.206	68.403	94.889	67.777
47.901	64.286	96.118	91.939
44.577	49.745	69.172	73.877
33.269	46.135	60.757	83.246
53.417	77.346	65.193	79.707
46.876	30.885	87.858	79.44
51.46	53.868	79.338	70.076
40.207	50.315	86.914	80.403
47.249	47.555	86.236	70.151
55.652	47.334	95.826	122.741
50.667	58.384	77.728	89.146
42.297	38.76	62.833	88.509
60.8	39.191	58.233	69.255
64.135	53.505	62.335	100.25
58.748	48.576	72.578	81.482
65.534	32.016	64.03	131.724
61.107	44.294	60.674	96.439
45.163	56.884	103.288	75.775
41.914	37.796	124.295	99.431

	33.878	28.432	86.548	68.138
	28.167	43.628	81.497	82.221
	49.334	30.59	99.428	67.582
	37.711	48.926	104.025	79.341
Mean		**44.415**		**82.596**
SD		**13.508**		**20.804**
SEM		**1.103**		**1.699**

Figure 9. 20H + Low Brain Fraction. 7h *in vitro*

Cell Pixel Intensity

22.9	23.36	12.243	15.622	37.839
41.081	49.095	32.167	16.006	18.786
20.635	22.736	25.661	18.997	24.031
55.376	36.525	26.774	29.898	30.96
48.839	18.206	15.411	29.68	28.797
24.836	29.852	21.903	26.094	17.4
26.842	21.577	16.405	10.03	34.496
39.615	17.932	24.837	27.438	44.418
34.457	13.643	19.147	22.905	47.29
27.109	30.602	23.99	12.81	46.719
26.074	23.047	23.543	18.662	32.909
55.93	42.028	25.446	28.304	33.643
27.487	45.253	13.747	24.778	38.097
46.741	73.548	23.63	35.943	31.241
22.576	19.688	12.535	20.615	37.588
15.124	21.142	16.462	23.984	35.717
15.793	21.637	33.116	14.173	27.827
23.995	20.305	19.932	14.713	19.702
25.471	60.67	27.013	20.619	24.865
26.104	17.706	19.488	40.831	14.802
49.872	19.461	18.626	26.585	25.594
28.11	28.018	16.102	45.189	28.324
18.024	27.402	17.878	40.748	30.818
28.925	35.476	22.746	35.151	18.009
20.799	20.969	21.874	34.759	28.47
30.179	14.969	13.077	49.514	28.451
51.401	14.986	17.905	31.172	44.344
22.513	17.723	18.027	32.301	35.316

16.536	12.74	32.061	29.324	36.637
31.759	16.216	36.516	33.54	49.009
			Mean	**27.682**
			SD	**11.179**
			SEM	**0.913**

Figure 9. 20H + Low Brain Fraction. 8h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
88.588	56.015	107.203	106.278
58.581	31.463	112.134	125.414
43.858	72.502	79.072	102.669
27.5	56.434	105.971	127.762
52.838	39.955	93.399	98.625
42.493	63.589	108.49	125.262
47.363	60.016	132.087	107.211
65.515	47.178	117.706	99.038
54.334	58.064	108.659	85.483
65.254	44.645	88.639	126.66
78.532	47.329	137.004	91.369
26.534	51.824	123.548	66.546
59.567	35.835	84.693	86.445
86.288	58.558	93.903	86.855
50.875	59.57	93.966	104.429
68.535	45.17	86.366	82.589
31.186	40.078	114.392	90.272
63.096	39.76	84.143	106.97
67.721	36.063	93.95	81.775
82.114	68.088	89.523	81.868
63.194	40.817	101.284	98.225
63.382	65.911	96.09	80.121
66.482	57.751	81.612	88.659
52.901	39.707	85.881	95.231
41.964	48.788	91.295	78.223
49.723	59.262	82.958	94.666
37.206	60.013	156.015	132.533
60.544	45.316	96.491	97.681
51.425	64.056	110.903	85.264
42.456	58.175	87.506	72.335

40.472	50.033	147.393	103.292
48.113	44.406	72.873	114.953
64.796	51.122	85.055	85.959
61.501	50.822	106.836	110.437
78.716	44.765	121.687	147.726
57.782	49.807	105.789	102.902
47.587	64.042	84.674	75.97
52.663	52.318	82.612	112.87
53.272	64.528	95.602	90.351
64.521	36.657	67.142	93.433
46.691	36.495	105.048	139.288
62.26	46.161	97.553	125.7
51.079	53.807	112.523	96.164
64.578	56.771	84.341	102.584
53.724	48.431	113.001	80.156
51.414	59.669	112.909	94.648
41.806	35.004	101.589	101.592
51.533	46.904	70.364	91.385
79.548	39.229	126.287	83.662
61.783	59.615	108.597	87.347
67.046	42.637	119.125	128.186
50.108	60.997	90.668	121.718
28.543	71.48	111.467	167.403
68.723	74.56	76.891	75.784
49.659	71.016	124.93	105.656
54.639	60.246	112.655	94.537
46.354	68.278	122.833	109.483
67.745	42.969	92.744	88.196
69.421	68.385	105.969	81.779
44.692	41.608	88.533	100.902
61.626	40.946	93.402	79.815
60.338	50.768	79.76	62.145
67.994	77.152	104.782	68.84
67.28	60.158	111.974	97.906
60.076	70.711	110.839	119.743
74.488	55.639	67.805	90.046
62.859	48.697	156.23	59.103
74.008	50.468	91.204	70.022
88.963	47.413	83.448	83.401
60.319	89.315	97.81	86.323

	54.606	59.017	83.135	76.762
	58.595	66.92	94.468	124.717
	64.14	75.02	87.508	112.772
	58.517	60.06	117.909	123.985
	51.03	57.563	73.571	135.73
Mean		**55.935**		**99.722**
SD		**12.753**		**19.926**
SEM		**1.041**		**1.627**

Figure 9. 20H + Low Brain Fraction. 14h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
75.453	50.365	93.098	128.622
50.667	44.675	63.839	111.7
44.316	29.463	53.876	93.218
59.26	48.599	94.626	89.699
40.72	76.074	106.837	116.604
86.291	98.205	102.009	95.34
39.13	63.901	76.487	45.097
29.148	59.157	58.142	56.511
22.87	84.717	72.304	39.671
28.917	67.558	84.647	51.405
28.942	82.535	67.291	69.578
36.633	95.502	87.939	68.272
49.671	127.856	78.573	114.217
61.898	56.701	107.704	59.957
38.58	65.418	52.506	78.781
41.796	80.037	106.266	70.525
39.701	41.978	102.188	73.647
43.038	68.963	110.445	58.098
41.324	64.121	100.057	56.081
76.845	45.734	129.436	63.371
58.856	50.057	81.637	58.54
56.409	54.329	100.146	110.598
72.456	57.184	97.565	74.969
72.33	38.333	91.265	87.169
74.441	35.28	88.682	101.975
63.935	67.788	115.802	112.55
101.34	40.97	71.637	83.192

106.902	28.463	84.74	113.664
85.483	24.452	65.524	91.618
105.616	37.81	49.646	66.652
68.331	44.038	108.249	83.887
74.753	42.65	39.035	81.86
63.686	61.562	99.299	83.612
66.18	50.834	107.77	81.04
53.835	69.364	111.886	91.89
69.389	95.6	89.292	84.723
75.051	43.383	147.731	52.186
39.082	32.755	97.052	112.108
77.875	52.155	62.072	63.097
47.026	58.213	145.858	114.55
45.998	56.099	135.425	133.169
53.119	64.215	102.854	86.126
53.347	61.668	85.262	94.976
62.584	38.54	154.703	113.05
59.952	55.03	129.394	106.454
56.233	38.882	109.531	114.077
67.455	45.556	105.932	94.085
46.775	53.041	89.569	89.449
81.824	76.671	92.119	42.753
58.182	85.754	117.227	51.464
45.19	92.602	94.854	51.155
55.649	83.035	81.191	62.898
48.345	53.055	75.121	42.765
68.402	60.119	82.981	52.573
49.693	87.139	117.531	60.62
70.295	49.85	65.207	59.713
70.5	63.135	58.389	53.174
58.319	77.45	81.722	52.119
76.549	60.635	41.581	53.418
51	77.194	59.435	55.358
71.237	61.685	90.748	50.536
68.69	62.703	121.213	73.17
50.669	49.668	59.842	45.857
69.806	42.006	126.123	60.571
72.254	72.492	116.283	69.977
79.936	64.78	131.155	73.385
88.231	65.864	86.22	69.937

	77.822	60.455	142.736	59.418
	45.161	77.812	116.988	67.775
	75.317	58.714	95.471	78.226
	48.067	67.602	92.14	86.74
	81.741	41.05	109.762	108.164
	83.64	39.419	91.333	86.02
	95.621	39.308	144.502	111.98
	93.359	59.141	87.369	87.795
Mean		**60.855**		**86.562**
SD		**18.719**		**25.987**
SEM		**1.528**		**2.122**

Figure 9. 20H + Low Brain Fraction. 20h *in vitro*

Cell Pixel Intensity

42.455	34.434	55.416	41.393	55.513
64.8	76.427	83.649	72.498	54.933
81.734	70.088	52.28	77.056	66.372
73.861	50.89	85.611	52.708	72.585
59.946	108.303	124.219	40.205	49.134
58.417	38.045	62.994	69.064	46.303
79.749	42.917	83.633	82.362	63.194
95.461	16.893	42.568	60.143	56.659
77.294	80.388	62.872	49.715	51.981
72.808	67.666	25.045	84.002	27.167
64.956	47.738	61.672	28.048	43.645
80.665	44.86	65.669	56.569	38.382
70.054	68.868	93.888	93.974	33.846
58.898	68.237	66.353	65.263	38.716
59.562	47.344	76.795	50.688	53.682
50.897	64.083	57.873	38.312	82.846
65.84	39.533	85.798	36.758	140.935
50.572	50.389	67.688	84.6	64.467
52.644	51.047	45.464	76.117	82.014
85.718	57.212	93.304	79.04	68.522
65.475	28.38	52.92	50.406	103.178
74.634	72.483	32.677	73.611	37.825
54.648	99.469	44.577	59.55	46.172
49.65	40.227	81.367	75.562	46.189
67.253	30.897	65.226	78.996	59.906

58.919	75.717	44.24	48.052	85.602
100.632	100.311	76.196	36.622	67.921
43.756	80.599	64.326	55.096	103.938
55.169	77.172	48.421	58.726	91.207
56.98	71.052	62.728	69.017	88.937
			Mean	**63.377**
			SD	**20.105**
			SEM	**1.642**

Figure 9. 20H + Low Brain Fraction. 26h *in vitro*

Cell Pixel Intensity

22.726	21.867	46.227	26.757	44.81
14.732	36.904	39.112	26.749	24.81
41.535	20.842	38.89	24.327	26.891
31.537	18.949	21.498	37.614	21.316
23.087	19.89	23.974	33.58	35.792
47.511	26.278	23.64	24.147	23.607
12.741	10.329	15.736	28.676	21.691
24.788	23.774	11.086	18.066	45.536
22.675	48.093	8.692	33.393	23.768
35.294	9.896	16.287	27.073	43.613
28.966	9.965	12.424	45.726	44.674
32.042	19.183	33.831	32.538	27.484
25.328	35.747	13.789	21.396	18.737
75.142	33.896	32.321	18.957	35.701
23.56	16.624	48.771	35.493	24.844
35.589	38.349	28.866	23.584	15.228
49.266	42.207	23.338	26.249	21.49
24.361	32.735	23.026	23.894	20.463
29.546	40.698	37.832	23.494	18.385
12.427	14.379	23.782	40.309	9.484
15.233	18.953	17.319	24.319	18.461
15.601	28.136	64.979	39.507	26.086
13.007	28.446	39.937	39.658	38.743
13.4	22.414	33.821	36.028	9.801
19.904	18.256	40.429	19.497	19.265
38.62	35.11	36.657	43.543	16.895
13.95	32.046	23.498	36.771	13.463
12.267	34.614	36.37	33.689	11.819
26.437	26.263	35.82	55.887	41.659

10.764	35.598	12.455	31.24	24.737
			Mean	**27.722**
			SD	**11.587**
			SEM	**0.946**

Figure 9. 20H + Low Brain Fraction. 32h *in vitro*

Cell Pixel Intensity

11.82	27.409	19.742	21.257	11.274
13.375	22.272	14.493	24.782	18.383
11.881	14.122	16.796	15.818	15.936
19.355	23.265	21.581	15.18	10.809
13.335	13.407	14.181	22.756	29.507
18.688	22.21	14.602	16.173	11.309
8.857	35.33	16.115	30.806	13.886
22.376	22.161	13.606	18.989	11.256
15.297	15.776	16.902	31.091	19.949
19.759	19.835	10.238	16.129	12.219
13.781	25.644	13.586	12.9	21.481
15.699	20.462	20.897	18.498	13.849
29.526	21.362	12.699	22.564	13.204
6.986	10.959	18.2	20.911	15.686
14.182	16.417	23.674	17.73	16.473
13.786	21.379	19.113	13.512	19.068
14.902	17.215	16.452	16.656	14.181
11.534	18.329	18.931	21.843	26.536
14.543	12.02	28.103	9.339	11.664
17.492	21.818	19.979	14.383	12.691
17.242	16.143	14.911	21.851	13.272
16.819	17.354	21.778	29.943	14.122
12.756	14.664	6.526	17.76	15.17
12.789	18.167	30.053	23.013	17.695
17.506	18.976	21.03	20.244	21.246
15.424	15.765	13.803	14.092	20.866
16.224	11.582	8.791	24.509	20.599
15.619	20.432	13.99	33.724	9.99
14.042	22.985	13.152	26.364	26.103
22.715	18.687	12.621	8.164	28.811
			Mean	**17.728**

SD	**5.503**
SEM	**0.449**

Figure 9. 20H + Low Brain Fraction. 38h *in vitro*

Cell Pixel Intensity

7.045	9.085	35.243	21.346	27.413
8.715	26.32	19.679	17.151	13.316
6.922	33.224	21.936	30.257	13.978
12.201	33.139	12.146	35.103	13.638
13.976	22.117	31.241	13.817	22.186
11.587	48.969	16.049	9.627	18.022
7.585	31.253	16.682	26.898	20.439
9.481	20.208	9.044	23.928	16.944
17.605	20.63	30.319	28.974	22.833
20.917	35.293	33.243	40.191	26.034
25.559	48.928	17.107	27.847	23.94
13.839	29.716	13.001	16.364	34.823
23.003	31.996	19.269	33.119	12.834
14.162	28.817	12.95	32.631	9.628
10.558	30.311	13.787	33.541	23.486
16.504	31.602	22.102	29.981	35.702
38.495	50.44	8.968	11.974	80.421
19.714	25.555	15.38	25.344	8.152
19.375	11.177	16.045	23.556	11.583
18.79	41.106	18.788	14.356	9.39
35.328	40.037	17.645	21.523	13.716
29.658	17.939	23.111	32.688	10.502
10.369	18.067	19.814	19.415	17.812
6.705	25.338	31.323	26.327	6.484
6.978	23.532	9.924	25.666	6.752
7	23.339	25.362	13.583	6.683
6.892	25.584	13.466	11.511	13.212
7.553	21.166	19.943	10.701	26.429
6.616	8.767	24.808	14.373	7.745
6.545	20.591	23.068	29.024	16.782
			Mean	**20.914**

SD	**10.954**
SEM	**0.894**

Figure 9. 20H + High Brain Fraction. 1h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
49.619	46.737	66.626	57.205
27.97	39.364	64.45	69.065
25.808	24.526	60.047	62.246
26.808	29.91	82.628	78.442
36.807	21.586	69.933	63.017
36.996	29.421	62.897	61.921
32.739	20.157	76.311	51.826
18.771	14.706	65.224	58.683
43.417	26.082	75.447	46.835
39.542	30.172	134.631	47.618
50.136	32.883	82.887	82.6
28.78	42.018	84.256	87.715
33.542	37.14	140.985	91.799
42.001	52.698	50.954	96.827
35.917	42.339	78.868	51.009
33.215	44.311	92.016	83.179
28.941	44.076	85.637	78.774
30.038	29.463	71.907	80.528
42.265	42.479	63.803	57.651
35.04	49.853	56.632	51.739
37.381	57.213	54.519	65.075
22.106	62.992	71.694	57.365
21.201	50.789	64.392	51.885
34.437	43.964	72.24	60.599
40.636	43.65	72.247	49.475
48.144	59.384	75.673	59.541
38.713	53.117	58.776	51.085
43.936	50.724	46.723	96.961
38.229	24.575	57.458	61.459
51.494	37.454	55.488	74.453

37.698	27.808	62.476	19.37
43.466	27.033	55.078	22.73
55.259	20.505	75.536	22.772
36.919	20.417	91.339	28.407
48.178	15.051	111.262	35.065
49.415	28.306	104.9	33.256
45.871	28.463	57.568	57.076
45.825	30.462	46.744	55.026
42.896	31.794	56.793	65.677
42.294	29.69	51.556	47.555
40.609	69.226	42.366	67.187
38.546	32.089	48.772	44.216
37.602	28.644	83.125	86.961
32.723	26.297	85.861	55.34
43.511	28.182	76.349	73.153
30.075	35.036	38.178	76.483
43.099	35.954	46.85	106.088
41.594	40.64	37.96	84.572
34.811	32.311	36.108	53.07
37.186	29.738	45.629	48.591
66.047	36.371	33.559	59.774
49.876	33.023	112.256	124.158
29.183	52.589	118.222	103.278
30.53	23.705	134.754	109.041
32.893	56.857	85.581	62.005
48.906	44.575	71.232	56.625
32.686	59.883	125.32	67.565
47.71	24.084	96.689	114.955
54.966	23.782	99.755	106.631
27.717	29.16	80.293	108.915
26.861	31.481	38.941	113.511
28.352	30.716	81.645	102.417
33.455	24.401	45.386	98.561
34.454	24.435	80.103	80.756
31.827	19.674	87.467	78.374
33.82	25.577	69.23	82.936
40.745	29.002	121.752	91.935
38.36	21.114	104.748	103.599
37.795	20.864	100.212	115.431
51.692	36.673	140.583	69.985

	31.558	24.695	115.173	52.237
	31.04	31.471	141.477	44.08
	28.434	18.097	81.648	130.126
	27.257	23.789	77.669	114.093
	32.049	23.774	67.201	92.373
Mean		**36.011**		**74.088**
SD		**10.784**		**26.355**
SEM		**0.881**		**2.152**

Figure 9. 20H + High Brain Fraction. 7h *in vitro*

Cell Pixel Intensity

16.275	30.924	15.675	32.325	9.707
22.572	9.137	14.17	26.654	14.913
17.726	22.745	25.257	23.403	13.365
22.745	26.719	17.63	22.615	11.097
19.002	22.745	24.968	18.236	19.413
23.424	28.662	20.283	17.606	15.81
12.009	36.546	15.46	44.899	15.006
23.608	26.594	21.187	21.018	22.694
16.298	21.045	20.267	19.415	23.849
21.754	24.656	27.278	24.137	14.848
24.203	36.342	17.026	14.023	14.748
45.742	20.599	22.613	13.979	25.312
27.957	18.504	25.456	16.318	19.229
23.107	21.317	23.384	24.621	29.057
22.355	40.958	15.354	24.654	39.348
27.646	75.524	22.256	17.023	22.285
21.545	19.922	15.882	13.624	21.434
31.279	18.496	16.668	14.42	15.522
16.652	21.112	13.362	24.522	18.743
27.285	16.774	21.553	26.333	15.642
17.076	18.31	19.215	15.564	21.798
19.421	19.057	23.366	22.922	18.641
14.941	8.602	29.129	13.954	17.325
15.87	20.16	14.039	15.596	11.741
19.976	15.056	19.3	14.231	8.932
11.451	30.501	12.969	9.616	21.743
19.921	33.43	14.063	18.042	21.126
17.083	22.697	21.219	14.322	12.358

17.53	21.508	40.327	12.7	19.779
12.031	19.118	21.286	12.079	14.496
			Mean	**20.838**
			SD	**8.158**
			SEM	**0.666**

Figure 9. 20H + High Brain Fraction. 8h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
74.142	45.846	123.947	96.643
49.161	59.571	123.267	173.225
80.066	35.191	117.736	121.492
50.803	48.298	96.31	70.286
38.709	50.689	97.167	88.676
48.079	65.726	115.257	109.863
83.727	66.711	102.1	93.85
66.398	56.58	117.933	88.323
92.972	52.169	140.247	96.703
33.811	32.119	89.928	131.161
85.051	83.972	106.386	126.137
71	97.593	96.687	105.994
74.307	35.745	102.975	126.175
89.055	69.083	89.371	115.783
70.165	59.578	90.701	94.81
72.758	72.447	112.059	81.185
113.903	53.931	89.251	108.029
69.921	61.596	80.143	131.447
53.482	66.654	105.402	108.939
92.227	87.664	122.67	83.467
50.227	53.57	158.415	97.233
106.282	75.937	83.092	67.716
73.969	39.898	74.848	85.218
70.837	64.458	100.43	72.351
52.797	67.598	100.032	92.887
75.926	63.354	87.083	103.74
63.904	89.264	91.917	100.71
89.171	72.739	105.415	132.224
78.747	55.057	99.19	110.942

89.978	65.95	87.171	121.551
75.909	53.082	117.885	116.484
78.756	50.016	115.135	104.246
78.488	57.214	87.363	115.818
83.546	106.155	90.278	89.618
37.394	57.474	91.833	94.466
93.576	101.605	94.506	103.3
52.247	59.019	99.361	125.045
53.98	114.63	96.577	120.955
76.592	58.264	116.906	143.116
50.667	74.31	100.223	109.003
73.619	71.078	108.099	118.578
79.559	76.63	96.736	198.773
34.479	40.772	153.992	108.576
68.648	81.132	133.558	114.48
62.659	54.631	134.398	99.029
87.44	29.953	131.923	136.052
93.857	53.548	138.455	108.453
79.434	82.477	107.372	147.277
30.879	39.639	72.373	133.638
90.647	71.894	90.381	108.252
73.182	95.435	76.43	120.531
70.049	96.575	78.635	162.526
102.469	89.647	73.854	123.952
86.816	35.755	81.167	123.953
49.983	84.886	146.537	90.931
56.114	37.707	143.096	67.577
113.53	106.847	69.667	75.855
95.024	71.208	104.055	89.579
49.408	90.361	114.242	105.071
60.926	86.122	145.356	126.922
64.241	74.558	150.516	126.446
91.585	66.08	88.349	119.357
60.869	94.149	123.675	148.242
86.845	72.346	138.296	112.661
100.365	85.111	88.994	134.812
97.079	76.101	126.945	118.833
68.867	67.184	88	115.24
57.048	82.757	88.185	130.467
47.718	61.063	87.086	145.182

	61.8	59.096	88.21	140.154
	65.763	47.23	84.812	131.309
	80.508	86.179	132.299	111.246
	63.751	73.19	144.434	119.765
	64.715	51.181	126.209	166.722
	73.051	38.946	135.103	169.974
Mean		**69.319**		**110.786**
SD		**19.253**		**24.022**
SEM		**1.572**		**1.961**

Figure 9. 20H + High Brain Fraction. 14h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
74.167	44.142	91.582	92.725
75.931	37.447	158.946	122.235
68.917	55.908	130.693	128.191
34.077	61.45	74.897	64.234
55.437	32.641	79.122	98.158
67.478	34.441	78.889	58.312
48.26	63.464	78.076	119.624
50.173	45.953	70.17	96.356
57.963	40.928	122.634	110.029
45.875	53.323	166.1	92.913
46.488	40.127	48.217	116.179
61.758	69.779	74.224	99.901
38.175	43.968	117.871	93.257
57.785	54.782	69.118	81.418
86.137	48.343	124.679	147.867
37.459	46.32	182.192	64.508
57.417	88.997	72.35	87.826
58.897	39.327	105.645	70.488
47.118	41.981	104.464	87.794
61.885	58.984	76.487	83.111
64.658	43.556	75.214	100.947
68.61	56.868	84.976	65.265
48.124	62.787	106.115	71.289
93.419	55.299	90.213	72.995
65.42	48.012	110.397	91.019
37.489	52.8	67.012	79.445
34.692	43.233	128.203	90.556

93.826	37.664	105.709	111.68
54.851	58.225	88.696	159.56
56.305	57.022	80.118	84.822
59.877	38.02	80.654	71.761
53.772	51.354	98.734	103.762
76.08	56.751	83.973	108.186
58.3	54.819	86.46	125.044
60.24	39.946	84.462	73.486
74.546	35.88	93.491	77.948
51.519	42.565	117.449	105.732
62.683	61.026	102.954	115.815
49.984	25.751	106.575	110.926
39.133	29.99	92.639	110.63
41.431	26.433	96.491	109.824
47.519	41.075	107.158	88.816
52.459	53.275	74.877	126.431
55.927	52.837	68.944	92.435
44.625	46.024	171.647	60.422
73.893	55.542	105.417	94.908
69.331	38.538	93.388	111.722
44.531	49.393	86.348	102.225
53.725	37.306	138.394	151.065
46.325	43.666	72.605	74.237
69.105	50.212	86.731	76.642
44.992	42.463	73.527	117.936
51.883	32.991	59.784	111.621
65.634	34.383	97.626	153.597
48.751	47.772	49.478	79.286
84.979	34.296	69.783	92.515
39.45	35.489	42.774	158.945
63.65	68.32	88.858	93.074
47.023	37.6	64.645	63.308
45.838	53.125	77.953	67.156
69.516	61.99	85.987	125.957
51.718	69.116	85.967	65.969
49.142	53.518	66.018	76.942
68.196	28.04	101.661	61.499
51.237	47.341	107.32	74.218
80.566	67.562	113.215	71.958
52	63.209	90.656	70.778

	48.719	60.05	81.545	98.823
	46.722	91.041	98.612	70.984
	31.24	44.12	84.507	44.164
	64.534	80.277	44.571	53.04
	55.103	51.82	99.734	41.463
	57.965	65.973	104.219	84.813
	59.934	30.377	79.734	52.897
	41.642	52.687	108.129	89.993
Mean		**53.04**		**92.962**
SD		**13.957**		**26.575**
SEM		**1.14**		**2.17**

Figure 9. 20H + High Brain Fraction. 20h *in vitro*

Cell Pixel Intensity

41.09	57.54	82.81	67.509	51.268
68	17.773	59.502	62.846	42.29
37.376	37.609	49.264	35.501	24.472
38.15	70.135	24.192	46.172	22.11
54.772	61.004	44.021	64.154	40.488
52.506	60.803	44.002	45.291	28.719
113.75	108.323	20.145	31.74	21.34
40.764	42.092	36.859	58.721	57.835
47.676	61.958	23.043	51.156	70.44
52.358	40.665	51.619	27.194	86.48
35.79	30.494	9.624	67.436	56.366
60.932	35.153	19.21	94.036	81.342
37.488	24.037	15.461	86.882	28.713
57.008	26.678	18.397	86.285	76.985
34.94	27.842	28.742	83.162	68.616
38.221	56.075	32.361	73.273	47.132
40.437	27.306	68.988	56.983	70.109
49.481	94.025	53.046	51.413	55.22
77.283	75.001	59.836	50.91	47.485
62.754	82.334	30.059	46.635	77.013
61.303	93.792	79.16	17.555	61.085
58.102	58.1	19.986	59.468	31.64
34.903	39.261	84.383	32.146	55.604
37.495	87.952	73.941	58.857	63.831
31.575	77.89	76.381	66.102	37.054

27.931	68.432	61.271	82.402	47.302
35.075	68.637	41.906	60.187	70.892
48.791	28.652	44.833	12.429	72.117
61.42	38.604	101.821	53.887	62.929
79.761	49.669	21.022	69.319	51.79
			Mean	**52.328**
			SD	**21.344**
			SEM	**1.743**

Figure 9. 20H + High Brain Fraction. 26h *in vitro*

Cell Pixel Intensity

12.335	12.023	15.35	15.338	17.192
19.784	17.728	25.436	17.395	22.088
19.497	22.632	19.977	23.123	20.452
17.657	15.193	16.233	21.293	20.423
17.721	13.493	21.56	12.07	29.855
18.217	17.861	18.722	13.02	25.06
17.828	18.719	22.854	20.347	19.606
22.579	16.381	22.028	30.979	17.198
26.654	15.292	12.266	20.796	15.688
19.338	15.66	36.462	24.053	23.581
23.002	14.273	17.509	25.052	13.546
23.083	19.8	15.698	28.164	16.266
16.297	17.937	15.056	19.914	16.044
11.883	19.405	13.22	12.595	27.996
17.975	12.894	18.819	15.216	11.377
16.703	19.919	19.278	17.068	32.763
18.156	18.722	33.466	27.635	17.293
27.337	18.066	17.005	23.803	12.798
25.355	24.668	18.908	20.141	19.382
16.188	25.491	17.908	22.705	14.637
15.187	17.103	14.057	10.64	11.741
16.518	17.641	17.561	6.929	16.91
7.856	12.337	23.58	12.722	19.581
15.153	20.491	18.847	18.291	32.422
14.099	30.983	22.324	19.327	24.977
7.567	23.995	16.45	16.398	14.639
15.749	17.738	17.712	15.816	25.028
10.007	22.96	18.457	22.367	20.413
18.485	23.727	16.761	15.235	35.055

13.622	23.281	15.58	11.401	27.416
			Mean	**18.991**
			SD	**5.423**
			SEM	**0.443**

Figure 9. 20H + High Brain Fraction. 32h *in vitro*

Cell Pixel Intensity

26.384	36.718	30.677	16.706	22.125
34.071	19.797	45.722	24.749	49.158
56.042	28.253	24.491	26.082	34.985
31.508	37.532	27.979	19.404	18.418
31.016	46.163	24.137	20.816	29.798
21.63	32.627	26.182	53.192	60.046
15.891	31.465	49.123	30.822	29.657
17.533	30.815	13.895	33.376	44.57
17.135	31.374	14.634	28.389	22.706
59.246	19.799	19.946	23.405	28.78
35.377	19.329	33.689	47.35	35.583
23.376	26.707	24.534	43.875	35.353
29.673	46.854	19.362	30.233	36.222
44.142	38.419	30.905	29.631	39.793
26.827	52.338	24.655	28.324	24.945
27.3	27.434	24.109	28.13	34.81
24.112	23.615	26.541	29.174	28.027
15.73	33.092	26.27	51.127	53.738
16.361	28.715	21.171	69.07	30.313
13.17	31.608	26.376	28.377	31.537
25.076	36.586	32.619	33.042	31.421
34.583	26.319	33.529	31.98	27.964
30.463	34.053	36.855	31.139	32.63
32.66	30.708	26.078	31.955	25.185
36.288	23.049	36.179	33.426	24.776
24.155	16.573	41.739	58.952	40.094
26.895	17.319	29.648	42.609	18.513
27.411	17.968	30.266	55.822	33.571
32.325	32.969	18.002	24.005	19.287

20.523	34.484	18.686	33.064	30.888
			Mean	**30.805**
			SD	**10.344**
			SEM	**0.845**

Figure 9. 20H + High Brain Fraction. 38h *in vitro*

Cell Pixel Intensity

20.743	13.562	25.211	56.207	32.656
26.907	118.606	27.567	16.284	36.251
38.79	20.053	26.127	21.077	47.099
35.821	19.188	34.999	18.286	45.179
58.66	15.022	31.08	11.725	34.642
13.397	20.74	22.859	28.681	19.602
55.862	22.26	21.772	35.911	22.1
21.148	26.456	16.404	12.726	26.428
28.696	12.82	19.959	17.152	10.369
12.176	16.693	22.635	30.902	22.258
13.373	29.642	13.618	29.831	20.188
9.504	24.704	35.736	35.92	15.405
12.235	21.973	21.9	14.728	13.677
14.942	14.808	14.405	23.831	24.565
19.866	29.253	9.541	63.031	24.541
19.007	35.698	30.267	17.953	16.268
14.458	34.795	21.244	19.986	17.109
27.212	44.205	28.229	16.235	12.474
17.435	15.403	21.351	15.117	25.542
22.897	35.446	20.822	17.973	24.112
16.615	7.383	22.423	23.573	21.229
20.636	8.356	17.725	63.969	13.989
25.992	15.72	13.596	16.851	11.218
17.839	16.961	24.222	18.115	18.195
14.746	15.794	14.735	18.411	14.931
14.48	23.669	16.702	11.453	17.629
22.919	9.335	23.339	34.217	26.302
19.626	18.058	37.143	21.2	15.368
24.209	8.165	29.376	19.405	30.687

14.519	6.968	31.224	20.915	20.822
			Mean	**23.368**
			SD	**13.087**
			SEM	**1.069**

Figure 9. Low Brain Fraction + High Brain Fraction. 1h *in vitro*

Cytoplasm Pixel Intensity		**Nucleus Pixel Intensity**	
51.5	72.149	92.299	81.755
35.524	58.588	100.6	81.776
49.592	33.065	49.052	89.426
78.076	30.284	29.348	78.177
68.111	38.879	53.51	54.653
50.674	38.187	47.433	53.709
65.526	28.089	63.091	72.496
61.499	56.956	61.545	52.113
84.132	36.709	60.412	57.312
56.68	50.665	102.589	109.137
109.376	59.561	103.974	119.763
82.555	29.237	99.401	97.634
51.569	32.442	92.183	119.195
51.718	40.431	84.524	75.537
59.57	30.991	89.455	116.098
50.637	61.092	86.19	134.102
47.256	49.977	105.672	111.741
35.656	39.055	69.655	109.032
56.232	54.009	76.11	152.103
29.712	45.307	78.225	154.776
32.14	45.604	75.693	132.184
39.343	53.453	98.475	89.222
45.35	50.539	92.658	87.342
51.353	48.44	87.93	117.251
59.776	52.655	95.386	85.278
61.71	63.943	93.28	116.473
51.512	60.114	99.338	93.743
51.115	68.622	72.556	104.93
47.027	54.449	95.894	83.375
52.685	29.165	108.547	120.513

65.21	70.178	86.752	57.091
54.819	67.063	91.123	53.508
64.139	32.686	113.699	54.981
28.025	47.378	112.48	58.193
57.103	40.416	81.524	74.876
54.572	53.6	109.8	50.046
41.183	39.292	149.043	128.379
51.117	41.481	96.787	108.378
57.804	61.251	105.46	85.873
51.101	54.46	105.516	106.688
96.502	77.81	100.908	126.515
84.634	59.41	117.328	112.234
79.078	67.163	111.366	95.539
31.207	57.408	100.416	53.225
109.864	56.341	100.231	57.209
75.241	58.793	40.8	73.536
83.237	52.321	91.267	73.496
62.853	58.911	61.608	58.553
64.647	61.731	77.517	70.75
53.151	47.972	61.373	71.471
48.898	64.573	85.967	69.425
108.148	57.243	92.054	101.836
59.679	39.322	57.322	90.434
75.464	35.7	85.036	71.818
70.186	28.397	84.009	85.298
36.627	33.487	77.707	99.667
50.841	57.251	62.189	78.936
71.463	60.849	89.46	48.032
21.932	59.955	82.336	42.132
20.994	66.936	94.33	44.284
28.104	45.611	122.402	64.629
68.788	53.13	119.073	36.896
59.558	51.827	127.47	41.417
65.326	55.934	87.048	45.609
47.965	48.436	131.472	48.112
72.079	42.58	81.059	48.862
44.27	68.36	134.119	54.048
71.508	45.296	80.24	66.474
40.721	74.049	147.678	81.969
85.843	44.547	108.874	57.36
60.186	72.118	88.726	55.419
49.671	61.083	86.248	55.036

	60.557	61.195	86.578	60.725
	69.972	62.194	91.671	83.335
	76.852	85.651	110.653	86.371
Mean		**55.285**		**86.222**
SD		**16.567**		**25.925**
SEM		**1.353**		**2.117**

Figure 9. Low Brain Fraction + High Brain Fraction. 7h *in vitro*

Cell Pixel Intensity

43.626	35.264	21.724	18.501	16.684
33.232	28.579	17.795	38.329	21.27
26.497	18.695	23.408	7.661	13.671
22.958	19.13	34.288	11.941	25.433
25.361	13.583	50.877	12.175	16.251
26.213	24.556	40.96	27.168	22.551
19.94	15.845	30.321	28.389	34.954
25.221	49.943	21.129	11.158	31.299
20.679	20.672	14.976	28.214	38.401
16.13	19.339	20.437	10.974	26.148
44.296	11.577	38.023	14.964	25.25
19.998	13.167	50.57	15.156	35.099
21.516	27.31	35.492	19.165	15.984
32.086	35.976	50.448	15.393	14.989
34.064	19.567	39.056	15.478	24.842
29.643	26.536	37.56	15.493	22.624
22.776	22.042	49.655	17.149	14.967
21.888	18.677	20.781	14.192	32.662
26.395	30.161	21.779	33.484	38.595
16.622	17.802	21.223	29.824	50.552
23.812	31.753	16.237	12.103	29.902
15.912	23.397	25.487	15.453	39.26
28.47	29.302	47.3	16.795	68.551
35.054	18.731	46.307	22.855	54.424
31.447	27.856	72.972	15.78	12.846
23.619	21.958	61.875	18.193	15.511
29.564	24.373	33.148	18.28	28.205
20.55	39.99	35.865	18.323	37.374
32.677	23.197	15.331	39.253	36.565
38.255	15.209	21.321	13.114	54.274

Mean	**26.821**
SD	**11.979**
SEM	**0.978**

Figure 9. Low Brain Fraction + High Brain Fraction. 8h *in vitro*

Cell Pixel Intensity

40.993	17.225	20.335	22.929	17.601
23.135	13.567	17.791	20.485	19.059
20.911	23.814	7.567	12.318	11.142
35.877	14.227	20.206	17.169	30.507
22.551	16.117	19.978	16.598	19.336
30.949	16.521	16.626	16.49	20.739
27.351	18.38	22.961	15.269	14.414
14.102	18.084	16.043	15.22	19.895
15.142	17.778	20.547	15.404	22.994
26.771	16.359	19.287	13.966	21.667
11.833	16.911	16.126	9.416	14.622
17.685	17.84	21.309	11.06	13.56
25.252	15.963	14.175	13.934	11.35
24.704	17.568	16.491	7.943	21.494
26.295	17.641	16.199	19.006	14.278
29.061	16.266	22.805	9.966	15.801
17.004	8.235	13.26	13.026	22.05
20.812	17.425	13.475	13.106	17.025
20.947	11.295	15.369	10.925	20.216
26.746	14.516	10.582	22.147	13.101
34.942	11.821	11.411	19.085	20.205
19.107	16.441	9.956	29.796	13.586
31.706	14.092	11.449	28.265	11.728
24.436	38.71	16.71	13.81	21.944
20.464	14.078	24.65	32.069	15.424
14.569	16.608	18.157	17.798	14.495
17.296	14.652	15.216	14.621	9.014
22.202	23.703	15.831	18.901	11.063
24.458	19.297	17.266	17.315	30.819
7.079	24.08	14.235	11.611	24.575

Mean	**18.286**
SD	**6.158**
SEM	**0.503**

Figure 9. Low Brain Fraction + High Brain Fraction. 14h *in vitro*

Cell Pixel Intensity

24.094	20.295	30.083	19.016	24.153
33.899	18.477	20.946	14.332	26.415
37.151	21.266	31	17.797	20.874
19.303	27.87	22.723	23.694	26.316
23.073	26.371	27.483	27.25	36.458
21.307	30.811	32.357	20.577	32.971
31.661	25.148	23.633	16.63	23.781
53.044	28.359	20.989	34.664	18.003
61.631	35.957	19.091	37.942	12.195
53.279	45.827	15.279	19.688	25.509
49.973	37.792	18.782	60.927	22.703
29.815	85.385	19.926	25.554	32.266
31.259	31.992	18.388	24.928	14.057
30.694	41.88	13.631	26.603	31.007
49.539	24.72	21.344	26.528	30.259
24.7	20.003	8.763	33.779	27.965
19.21	24.476	13.164	19.031	19.254
42.037	23.239	16.174	24.6	35.386
12.529	23.671	20.782	28.992	38.383
12.979	18.661	23.694	18.294	25.07
17.328	18.629	26.071	19.077	40.025
46.055	16.3	20.493	25.647	24.963
23.093	26.686	17.2	24.5	23.304
47.411	11.415	25.77	21.712	28.841
20.894	24.169	20.515	22.491	29.479
23.768	36.714	16.393	18.892	41.348
44.299	23.755	22.583	37.637	36.542
36.646	35.408	35.176	30.924	72.105
15.131	15.578	22.284	50.612	50.882
32.078	21.964	19.276	23.494	24.511

Mean	**27.637**
SD	**11.675**
SEM	**0.953**

Figure 9. Low Brain Fraction + High Brain Fraction. 20h *in vitro*

Cell Pixel Intensity

49.069	37.299	19.155	36.482	54.516
21.936	48.064	26.033	10.939	34.372
40.378	23.739	17.401	48.342	52.802
41.294	31.045	32.717	50.135	47.055
32.383	42.845	23.141	31.088	71.193
38.984	34.117	28.079	52.482	60.696
36.696	64.433	47.599	28.694	45.771
48.192	20.02	31.875	34.938	61.999
32.702	35.605	36.512	12.635	69.069
36.121	33.116	48.824	19.961	14.102
49.881	21.276	49.655	51.585	50.902
39.444	15.687	40.588	61.596	25.413
43.459	44.55	57.496	42.763	26.151
33.767	33.789	20.164	44.493	57.532
50.86	34.147	45.506	39.027	71.112
50.507	43.05	31.704	30.589	38.491
35.847	53.423	48.62	37.506	39.097
54.553	25.51	32.922	60.622	52.454
49.064	27.364	60.344	50.889	26.565
50.959	50.758	44.174	52.198	10.933
41.918	54.525	35.695	29.768	53.447
68.693	41.046	29.617	49.404	48.8
59.535	28.935	46.875	41.365	52.01
89.05	33.929	51.974	46.79	45.974
57.13	63.025	47.753	52.347	39.242
39.518	18.908	31.808	31.936	29.666
60.497	38.49	44.991	47.807	58.785
67.225	24.752	46.173	55.857	35.225
42.767	42.4	51.929	28.731	16.874
39.586	18.57	67.463	24.187	47.847
		Mean		**41.51**
		SD		**14.106**
		SEM		**1.152**

Figure 9. Low Brain Fraction + High Brain Fraction. 26h *in vitro*

Cell Pixel Intensity

32.866	21.539	50.741		35.911	19.634
10.985	33.038	32.822		37.069	13.702
12.597	23.846	19.612		19.891	9.135
11.571	28.255	22.208		23.225	15.908
19.526	54.16	33.028		21.627	22.68
14.728	26.763	29.556		35.061	25.525
28.542	21.96	17.498		23.917	19.089
17.811	39.27	21.24		21.95	30.068
26.936	17.029	7.525		37.082	29.145
21.041	33.299	10.91		26.321	22.075
25.128	10.599	11.759		33.665	34.98
39.463	18.987	8.836		42.703	18.798
25.854	19.101	25.699		22.181	12.756
33.246	23.562	34.482		23.747	16.365
14.805	28.894	31.401		23.29	28.039
12.161	12.629	32.613		16.309	28.748
32.389	18.75	34.919		20.89	22.352
25.269	32.907	25.818		16.796	14.395
16.594	24.04	21.915		19.369	11.344
32.787	29.298	11.604		32.855	22.611
32.773	46.658	19.901		27.451	14.066
16.687	22.775	16.359		28.247	34.614
27.696	26.158	27.972		26.981	21.781
29.051	32.622	18.391		30.334	20.929
36.763	32.174	17.613		28.391	23.248
31.288	24.179	26.929		25.317	21.678
41.882	29.831	35.153		34.784	21.044
44.808	30.35	22.371		39.251	22.963
27.122	26.216	15.157		22.938	21.925
29.562	21.085	31.283		19.972	23.855
			Mean		**25.055**
			SD		**8.613**
			SEM		**0.703**

Figure 9. Low Brain Fraction + High Brain Fraction. 32h *in vitro*

Cell Pixel Intensity

9.784	16.683	36.372	44.923	33.442
12.332	30.561	48.321	33.101	7.614
24.508	33.105	27.645	37.77	13.699
28.339	53.704	47.77	54.84	43.832
39.861	44.006	29.873	22.861	43.694
33.562	38.184	7.649	35.027	29.642
37.953	37.875	11.986	59.222	38.733
47.266	44.265	20.156	6.706	37.718
38.163	35.178	30.662	10.539	38.538
23.578	38.914	41.501	31.935	32.644
27.275	19.349	10.215	15.8	28.199
16.324	21.053	6.013	22.134	32.155
40.304	13.094	61.302	30.145	39.852
32.757	33.066	20.676	13.826	46.673
37.66	32.613	9.07	5.65	51.858
51.175	46.909	26.894	21.033	35.695
31.574	7.811	34.263	32.324	35.357
23.468	5.698	32.35	43.118	35.106
21.789	4.77	37.303	38.503	56.785
28.43	6.065	47.373	17.263	56.909
50.024	5.02	30.891	37.002	43.873
26.512	6.015	40.532	37.669	42.728
35.731	14.728	36.396	46.129	36.929
32.889	34.396	26.873	33.625	39.871
50.156	10.777	31.97	54.878	34.315
16.055	4.301	24.66	42.827	50.78
11.46	6.37	38.771	40.101	62.107
38.217	6.28	45.594	59.898	48.239
54.63	6.948	55.48	41.668	51.423
53.36	8.906	32.76	26.042	27.974
			Mean	**31.774**
			SD	**14.759**
			SEM	**1.205**

Figure 9. Low Brain Fraction + High Brain Fraction. 38h *in vitro*

Cell Pixel Intensity

21.6	12.106	5.726	12.842	20.437
18.825	24.002	5.419	9.568	17.533
25.11	20.979	6.49	9.96	22.544
16.848	5.906	5.711	17.807	16.8
20.183	6.075	5.785	20.587	19.461
35.209	6.803	7.216	19.819	21.851
20.24	6.564	6.398	16.214	16.625
9.002	13.366	5.1	11.518	14.393
24.298	52.224	11.082	9.591	8.367
29.596	6.3	8.214	15.269	23.789
18.2	5.737	6.896	20.678	19.648
24.81	8.041	8.108	14.946	25.088
12.052	9.33	7.11	18.419	29.428
7.554	7.84	22.477	13.796	44.729
12.33	17.107	23.248	9.737	26.392
25.899	21.096	17.531	11.74	9.771
31.626	20.32	14.176	13.572	9.603
28.724	4.916	12.554	13.95	5.863
16.186	5.012	8.172	9.299	12.621
24.781	17.204	20.499	29.274	8.429
12.464	14.446	19.091	14.211	6.359
10.939	5.345	16.017	17.504	13.376
18.821	5.439	20.376	16.18	20.652
13.532	26.193	10.942	20.665	18.121
15.951	9.046	19.06	15.168	15.481
13.978	5.899	25.931	14.549	27.971
15	6.124	10.313	18.908	19.78
12.869	5.471	22.227	16.602	14.324
19.694	22.35	10.538	14.985	7.953
24.186	18.333	9.325	17.677	9.21
			Mean	**15.556**
			SD	**7.846**
			SEM	**0.641**