FULL REPORT

ISN-CAEN Award

Visit by the applicant to another laboratory (CATEGORY 1A)

Project title: THE CIRCADIAN CLOCK OF RETINAL CONE

Host Laboratory: Dr. Marie-Paule Felder-Schmitbuhl

Université de Strasbourg, France

Dates of Visit: May-June 2015

By

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Mahidol University, Thailand

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Committee for Aid and Education in Neurochemistry (CAEN)

THE CIRCADIAN CLOCK OF RETINAL CONE

Background of the scientific project

The different forms of inherited retinal degeneration and environmental are characterized by the progressive death of photoreceptors (PR) rod and cone type. This visual impairment affects millions of people worldwide and its incidence is increasing in Western countries. The cones are responsible for color vision and visual acuity strong discrimination. Their dysfunction and degeneration are the main reason of visual impairment in humans. Yet virtually all pathophysiological data available concern the death rods, because animals commonly used for laboratory experimentation (rats and mice) have retinas composed mainly of rods (> 97% of PR). The pathophysiology of the cones is so far poorly characterized.

Invalidation of the gene encoding the transcription factor *Nrl* mice (*Nrl -/-*) causes a loss of rods and cones development of supernumerary (Mears et al., 2001). This cell line therefore represents a unique experimental tool for the analysis of cones which has already proven useful for the genetic and physiological characterization of these cells (Daniele et al., 2005). This mouse strain is present in the laboratory and we therefore have a relevant animal model to study the biology of the cones and to test potential protective strategies.

Living organisms are subjected since the beginning of time to rhythmic geophysical phenomena (rotation of the earth on its axis and around the sun). To survive, they have developed coping mechanisms anticipating changes in the environment, known under the term of Biological Rhythms. In fact, all the functions of an individual, whether physiological, endocrine, metabolic, behavioral or psychological, exhibit daily rhythms. These rhythms depend on circadian clocks that make up a complex multi-oscillatory system responsible for the internal coordination necessary to time the functional integrity of an organization. Oscillations of gene expression controlled by so-called transcription factors specific "clock factors" (*Bmal1, Clock, Period, Cryptochrome* for the major components) are responsible for the genesis of rhythms. These factors interact in negative autoregulation of loops, while causing the transcriptional programs the cell to a base 24 hours. These mechanisms are present in many tissues and organs (peripheral clocks) and are mainly synchronized by a central clock located in the suprachiasmatic nuclei of the hypothalamus (Okamura, 2004).

Rhythmicity is at the heart of visual function as it allows the retina to form images both night and day, despite considerable differences in light intensity (Green and Besharse, 2004 Iuvone et al., 2005). It depends on an internal clock to the retina (Tosini and Menaker, 1996) but still imprecise localization (Tosini et al., 2007, this laboratory, unpublished results) and controls various cellular processes. Thus, the visual pigment of the rods and cones show peaks

of expression at a particular point in the cycle (von Schantz et al., 1999, Sakamoto et al., 2006). Similarly, the melatonin and dopamine have rhythms in phase opposition, melatonin is produced at night in the retina, while dopamine is the day (Nir et al., 2000). At a more integrated level, the treatment of light information is itself rhythmic, which is manifested by changes in the electroretinogram during the 24 cycle (Manglapus et al., 1998, Tuunainen et al., 2001, Barnard et al., 2006) and results in visual sensitivity difference between day and night (Bassi and Powers, 1986). The clock also regulates processes directly related to the survival of photoreceptors, such as metabolism, phagocytosis of their outer segments (La Vail, 1976; Grace et al., 1996, Bobu et al., 2006, 2009) and their sensitivity to the phototoxicity (Organisciak et al., 2000). Finally, it is important to note that the process known to be regulated by the light in the retina, also owing to a functional circadian clock (Storch et al., 2007).

Although their physiology is not well known, it is clear that the cones take a key role in the adaptation of the vision in the day / night cycle. Main vision support during the day, the cones are also vital function at night, by secreting melatonin, a hormone that promotes local action adaptation of the entire retinal physiology for night vision (present laboratory results unpublished). The renewal of photoreceptor outer segments is an extremely active process to regenerate constantly phototransduction machinery, altered after light stimulation. This process is highly rhythmic, with a very marked peak in the beginning of day. We showed that the cones rich in mammals cones (Bobu et al., 2009) but also in *nrl -/-* mice exhibit a daily rate of phagocytosis of outer segments is retained after keeping in the dark (Krigel et al., 2010), confirming the one hand that the physiology of the cones is regulated by a circadian clock, secondly that the mechanisms of this regulation can be studied in the *nrl -/-* mice.

Objectives of the scientific project

Rhythmic processes occur in all cell layers of the retina and are regulated by a circadian clock of poorly characterized localization. Our results indicate the presence of a clock in each cell layers (Jaeger et al., 2015), especially in photoreceptors (Sandu, et al, 2011). The layer of retinal photoreceptors being composed of cones (3%) and rods (97%) mice, we developed the use of *nrl -/-* mice in which no rod is formed, in order to precisely characterize rhythmic process cones.

The purpose of this research project is to assess the presence of circadian clock specifically in the cones, an issue that had not yet been asked for technical reasons, and to characterize the molecular mechanisms. It also seeks to understand how this clock adapts to the day / night cycle and how it controls its target genes that will be instrumental in the cyclic operation of the cones.

Scientific and technical program

1) Identification of the clock cones

To assess directly the presence of a circadian clock in a tissue, we use bioluminescence technique which allows to measure in real time the expression of a clock gene in cells or in tissue culture. We currently have various transgenic lines in which luciferase reporter gene is controlled by a promoter clock gene (Yamazaki and Takahashi, 2005). In this work we use the mouse *Per2*-luc, in which luciferase gene was introduced in frame to the 3 'end of the coding region of *Per2* gene (knock-in; Yoo et al., 2004). The expression of *Per2*-Luc fusion protein thus reflects exactly the transcriptional and translational control of the endogenous protein and *Per2* present in all cells, the same oscillations. In the presence of the substrate luciferin, any tissue explant taken from such an animal and having spontaneously oscillations *Per2* gene generate a light emission itself rhythmic recordable over time. This tool is particularly suitable for the demonstration of the oscillating properties of a sample and to characterize its synchronizers factors.

We crossed mice from *nrl* line -/- with Per2-luc mice to generate *nrl* -/- animals, *Per2*-luc in whom the rhythmic activity of the cones can be followed by bioluminescence. With the first *nrl* -/- animals, *Per2*-luc generated, we have cultured whole retinas and analyzed their rhythmic behavior: the oscillations of the circadian clock are supported overall but less robust than those of wild-type mice (Figure 1). To isolate the layers of cones, the retinas are dissected and mounted with gelatin and tangentially cut using a vibratome (Vibratome 1000 classic, Warner Instruments). These photoreceptors layers are cultured on semi-permeable membrane and in the presence of luciferin, and gene expression *Per2* monitored in real time by the emitted luminescence measurements at all of the explant (Lumicycle, Actimetrics). The team commonly uses the technique of isolation vibratome that is conducive to the survival of cuts, to analyze the rhythmicity of the different layers of the retina individually (Jaeger et al. 2015). Thus can be evaluated the ability of cones, isolated retinal context, generate autonomously rhythms. To assess the importance of the cellular network and contacts between cells, the ability of the cones to generate circadian rhythms, we also isolate the cones by enzymatic digestion and put them in culture in the presence of luciferase. Bioluminescence is measured as before. If we can thus show the presence of a clock in the cones, we use the same type of explants subsequently, to understand the photoreceptors synchronization mechanisms. Thus, the role of light in training the clock photoreceptors (cones and rods) will be analyzed by bioluminescence, subjecting explants prepared as above with day / night cycles or flashes of light before or during measures.

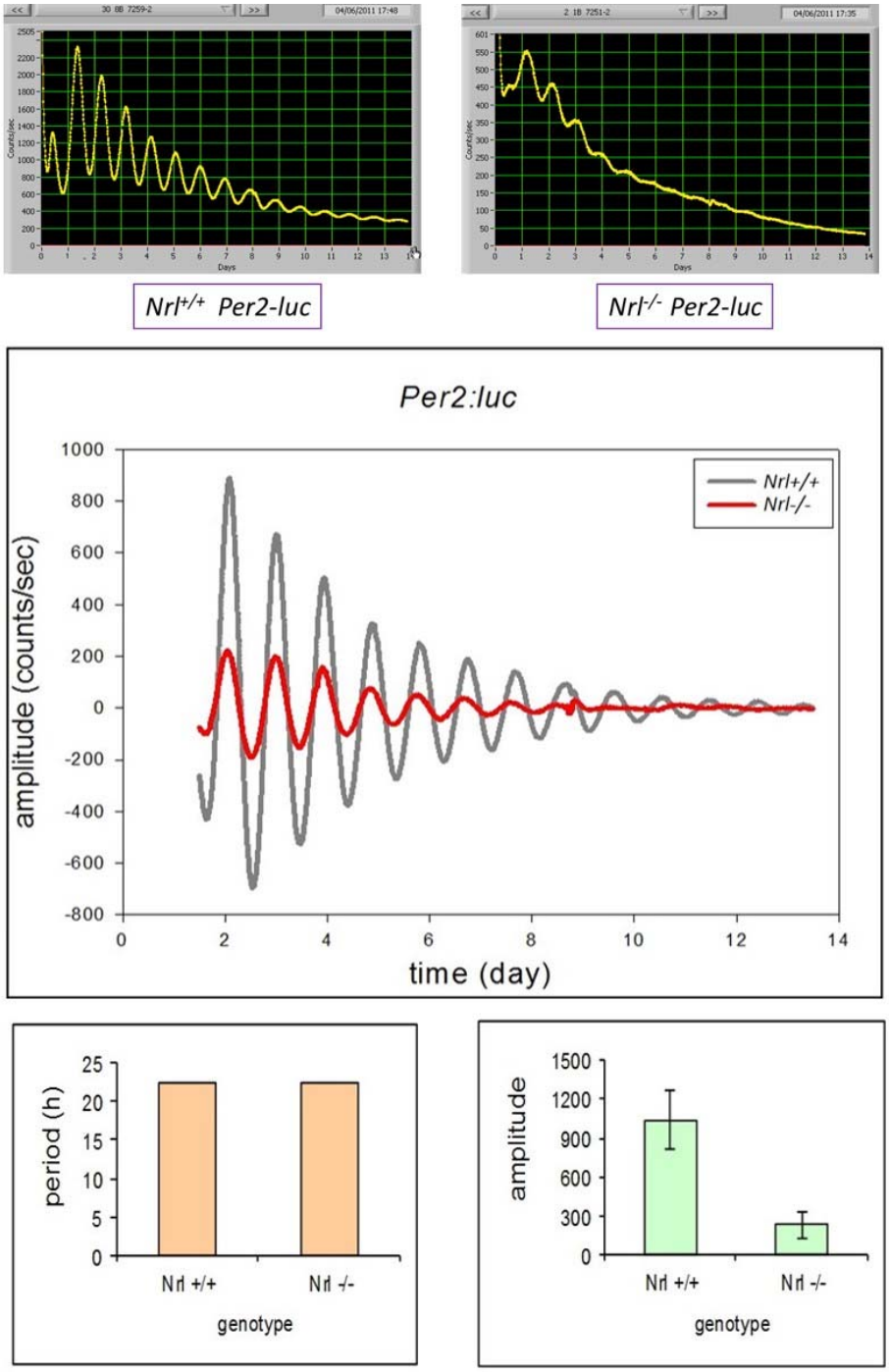

Figure 1. Evaluation of the ability of retinas containing only cones, to produce a rate circadian clock gene expression in vitro *Per2*. The expression is measured in real time by bioluminescence with luciferase reporter gene introduced into the *Per2* gene, in the genome of the mouse. The result (representative of all samples tested) shows that the retina contains only cones is capable of generating oscillations of the clock *Per2* gene but with a reduced amplitude and stronger damping. This result suggests that the presence of rods and cones is required for the clock of the retina works best

2) Characterization of the clock cones

The strategy used to study the clock cones from a molecular point of view, is to characterize the kinetics of expression of clock genes and 24h of their target genes in mouse photoreceptors layers *nrl* -/-. In a similar approach we characterized the mechanisms of the clock sticks in rats, and showed the importance of specific clock genes in these cells (Sandu et al. 2011). The kinetics of expression will be performed on 24 hours of total darkness. Indeed, the persistence of rhythmic processes in the absence of day / night cycle is one of the criteria to say that a function is controlled by a circadian clock. Thanks to the result, we can offer a model of regulation of clock genes between them, specific to the cones, and understand how they train their target genes and rhythmic functions of these cells.

Sample collection has already been completed, to the preparation of RNA. Specifically, animals (*nrl* -/- mice) previously raised in classic dark light cycle 12h / 12h, were placed in constant darkness for 36 hours and then sacrificed every 4 hours (n = 5 per time point) during a cycle of 24 hours. It is known that the *nrl* -/- mice develop degeneration of the photoreceptor layer in adulthood (Wenzel et al., 2007). This is why the animals used in our experiments are aged 5-6 weeks, so that the retina is mature but far from the stage of degeneration. The eyeballs were removed and frozen. After cutting globes cryostat (20 microns), the cones layers were immediately isolated by laser microdissection-capture (Arcturus, ALPHELYS). The samples were then lysed and RNA extracted (RNeasy micro kit, Qiagen) and then stored at -80 °C pending process all samples. The quality of extracted RNA was carefully evaluated by spectrophotometry (NanoDrop ND-1000 Spectrophotometer V 3.5, Labtech), in particular the ratio OD260 / OD230, and by microelectrophoresis (2100 Bioanalyzer, Agilent Technologies). Samples of RIN (RNA Integrity Number) greater than 6 were used for the next step; synthesis of first strand template complementary DNA and synthesis of amplify RNA (aRNA) (C&E ExpressArt mRNA amplification Nano kit, Amsbio) and synthesis of cDNA (iScript^{TM} Advanced cDNA Synthesis kit for RT, BioRad). The expression of clock genes were then analyzed by the PCR technique in real time (PCR 7300, Applied Biosystems) which is commonly used in the laboratory: TaqMan strategy with primers and probes manufactured and validated specifically for the amplification of messenger RNA (Applied Biosystems). The genes analyzed are; *Bmal1, Clock, Per1, Per2, Per3, Cry1, Cry2, Rev-erbα, Rorβ* (both a clock and a regulator factor of differentiation of the rods and cones, Swaroop et al. 2010). We have already done preliminary tests expression showing that all the clocks genes are expressed in the cones (Figure 2).

Alongside was studied genes potentially controlled by the clock cones, such as; *opn1sw* (opsin S) *opn1mw* (opsin M) *nat4* (encoding the arylalkylamine N-acetyl transferase, AA-NAT-limiting enzyme in the synthesis of the melatonin), *c-fos* (immediate early gene regulated by light and clock in photoreceptors) to highlight their rhythmic expression and to offer, through the analysis of their promoter, regulatory mechanisms by the clock.

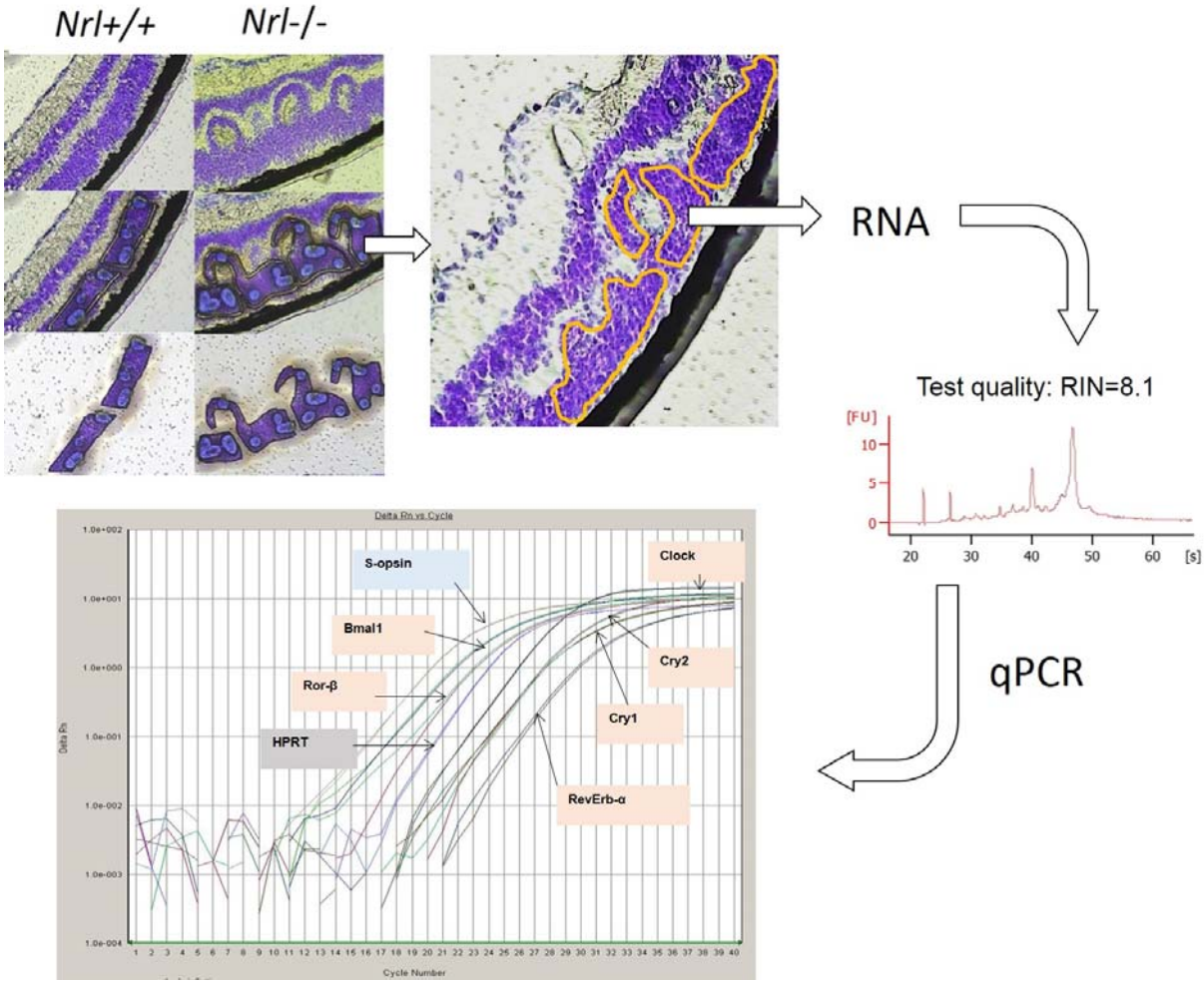

Figure 2. Isolation cones layers by laser microdissection (LCM) from mouse retinas cuts procedure *Nrl* -/- (tissue fragments collected are surrounded orange line). After RNA extraction and test quality (quality indices obtained are RIN 7 to 8 on a scale of 0 to 10), gene expression is measured by qPCR. Here we see that most of the clock genes are detected, as well as control the *HPRT* gene. The expression of S-opsin cones specific layers, is very marked.

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Outcome and benefit of the award

First of all I would like to thanks ISN-CAEN committees that awarded me the visiting grant to participate at CNRS UPR 3212 Institute des Neurosciences Cellulaires et Integratives, Université de Strasbourg, France, under supervision of Dr. Marie-Paule Felder-Schmitbuhl for 2 months during May-June 2015.

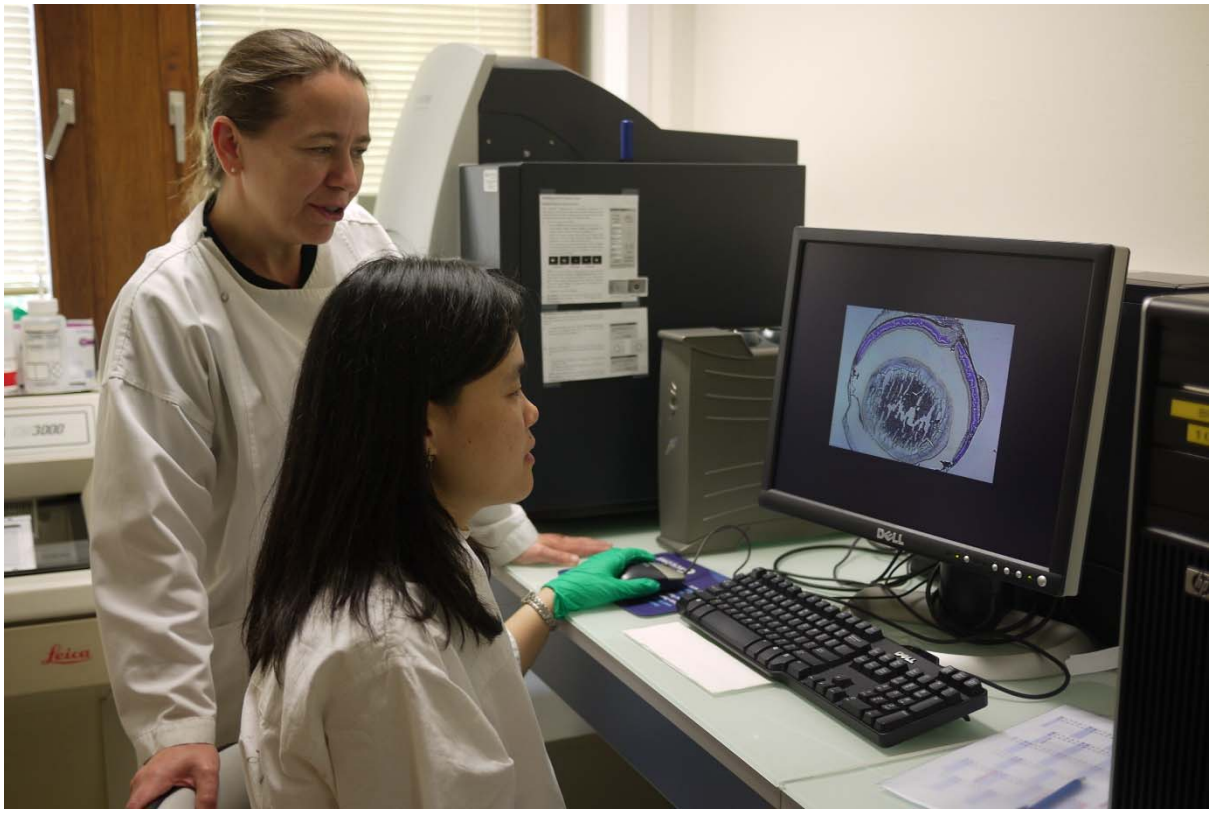
It was a great opportunity for me to have a change to learn and train a new technique of dissecting a specific or interest cell type that is fixed onto the slide by using a typical equipment called Laser Capture Microdissection (LCM, Arcturus Veritas) under the scientific project that has reported above which aim to study the expression of clock gene and clock output gene in *Nrl* knockout mice. This project also supported by the University of Strasbourg (USIAS) grant. I have learnt to sampling an eye, dissection, fixation processes, slicing an eye ball by cryostat in a specific manner for LCM methods which must to attached the tissue on metal frame slide and also have to stained in a specific process under RNase free condition. After cut the cone cells by UV laser then collected all samples to next step of amplify RNA. This method was use to generates more yield of RNA material by using a molecular technology by synthesize cDNA template that contained T7 promotor and then synthesize aRNA from cDNA template. This method is very useful for a sample that has a limitation of a source of material and also can produce very high quality of RNA for the next step of qPCR or microarray analysis. I have trained for the qPCR methods and the method to analyze the results by using several normalization genes (housekeeping gene) which very important nowadays for analysis and validate the mRNA expression level of target gene. Moreover, I have learn how to analysis the length of period and the amplitude of gene expression by using specific of mathematic equation to study the signal form transcription step of *Per2:luc* in the whole retina culture and photoreceptor layer which is a very new for me and it is interesting. The preliminary results from this research based project training that we get have a good trend to continue and we hope to get a significant results to be publish in the future.

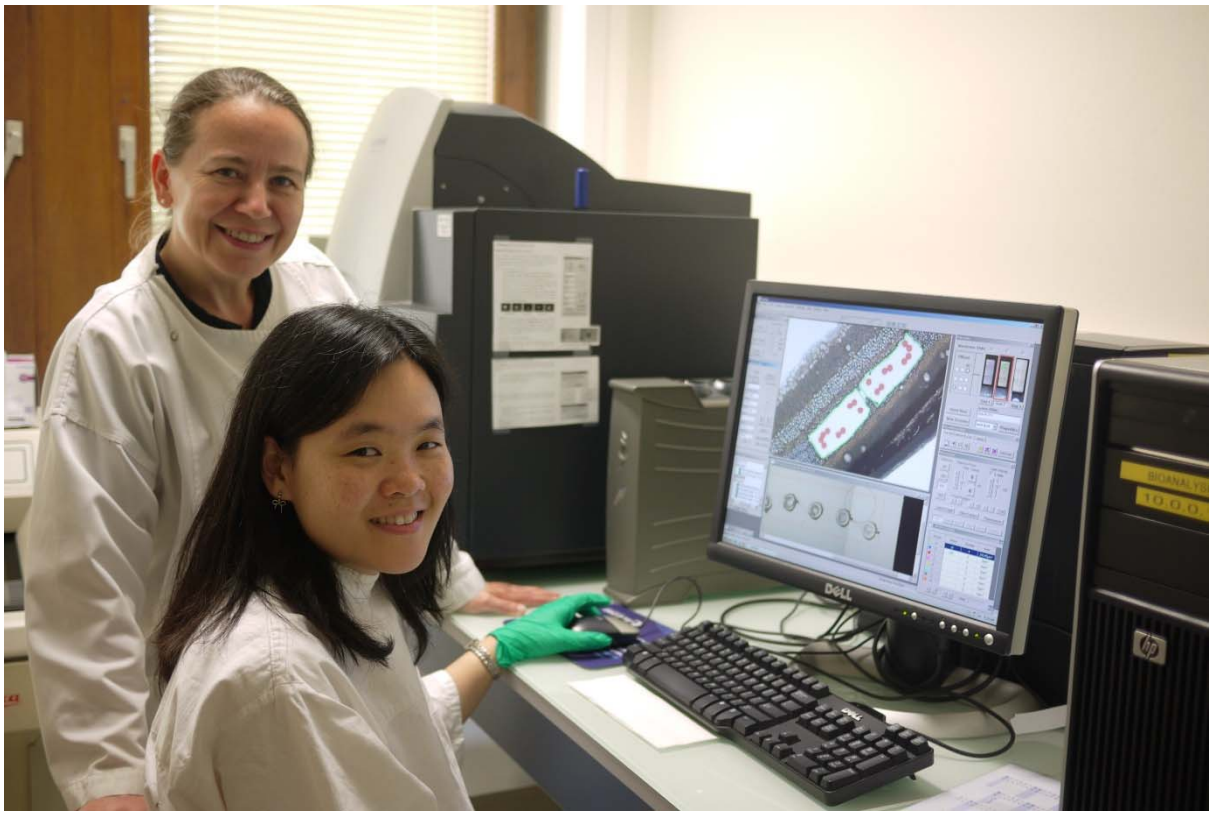
I have gained a lot of the valuable knowledge and experiences that correspond to my current field of research which will be of great benefit to my work and the faculty. I had a chance to present my university and work in Thailand to member of the NBR department which I have got a lot of suggestion and idea to improve my research. Finally, not only the scientific knowledge but I had a chance to visit Strasbourg and the town nearby and also joined and learnt more French cultures by many activities that happened in the lab during my stay.

Photos

Me and Dr. Marie-Paule Felder-Schmitbuhl

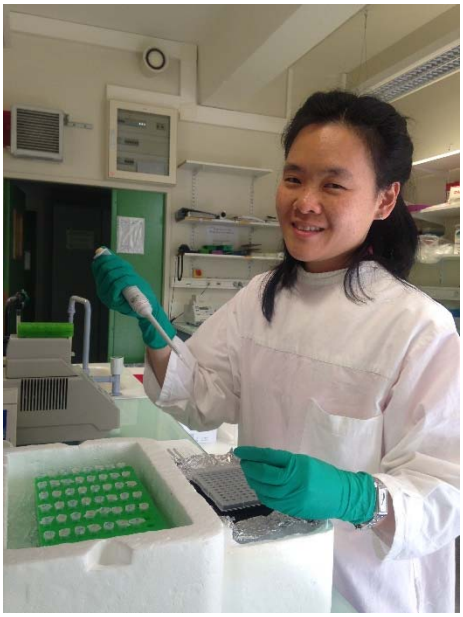
NBR: Monday morning meeting





Laser capture microdissection

In the Lab



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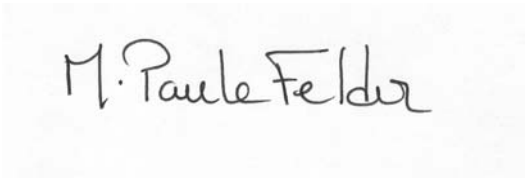
To whom it may concern,

Dr Prapimpun WONGCHITRAT has spent 2 months in 2015 (May and June) in our laboratory at the Institute for Cellular and Integrative Neurosciences in Strasbourg as an invited researcher. For this visit, she was also awarded a "visiting grant" from the International Society for Neurochemistry. During her stay she took part in our project to characterize circadian clock mechanisms in the distinct cell types of the retina. She more particularly analysed retina cones by using the *Nrl*-/- mice, a mutant in which the retina rods are converted into cones. She used laser capture microdissection to isolate photoreceptor layers from their retinas at distinct time points of the 24h cycle and extracted mRNA to finally analyse kinetics of clock gene expression by qPCR. We have been fruitfully collaborating with Dr Wongchitrat for several years: she is an excellent scientist with very good technical and conceptual skills, always eager to learn new techniques, whenever necessary for her project. She is also a very kind person, with whom it has been agreeable to interact, whatever the context. The topic she has been developing here this year is rather close to the projects she is conducting at Mahidol University in Thailand and I can tell that our interaction is mutually beneficial. I strongly hope that we will be able to maintain this collaboration in the future.



Marie-Paule Felder-Schmittbuhl, HDR