Zebrafish circadian clocks: cells that see light

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Abstract
In the classical view of circadian clock organization, the daily rhythms of most organisms were thought to be regulated by a central, ‘master’ pacemaker, usually located within neural structures of the animal. However, with the results of experiments performed in zebrafish, mammalian cell lines and, more recently, mammalian tissues, this view has changed to one where clock organization is now seen as being highly decentralized. It is clear that clocks exist in the peripheral tissues of animals as diverse as Drosophila, zebrafish and mammals. In the case of Drosophila and zebrafish, these tissues are also directly light-responsive. This light sensitivity and direct clock entrainability is also true for zebrafish cell lines and early-stage embryos. Using luminescent reporter cell lines containing clock gene promoters driving the expression of luciferase and single-cell imaging techniques, we have been able to show how each cell responds rapidly to a single light pulse by being shifted to a common phase, equivalent to the early day. This direct light sensitivity might be related to the requirement for light in these cells to activate the transcription of genes involved in DNA repair. It is also clear that the circadian clock in zebrafish regulates the timing of the cell cycle, demonstrating the wide impact that this light sensitivity and daily rhythmicity has on the biology of zebrafish.

It does not seem that many years ago since circadian biologists saw the world in a very centralized way. Following the initial description, in mammals, of the SCN (suprachiasmatic nucleus) as the central circadian pacemaker, many years of work had built on and confirmed this initial vision of a highly centralized, master clock [1–3]. The clock was in the SCN, and photoreceptors within the eye conveyed the critical light information necessary to set that clock [3]. In lower vertebrates, circadian pacemakers were clearly shown to be present in the eyes and the pineal gland, both being directly light-responsive in fish, reptiles and birds [4]. In invertebrates, such as the marine molluscs Bulla gouldiana and Aplysia californica, clocks were present within specialized neurons within the eye [5]. In the ubiquitous Drosophila, the circadian pacemakers were within the ventral–lateral neurons of the optic lobes. It was clear that ‘you put your clocks under the lamp–post’ or at least in areas where they could be easily set or entrained by the environmental light–dark cycle. These structures were typically neural, which gave us all comfort in knowing that we were, if nothing else, neuroscientists.

But times change, and we now live in a decentralized world. The first moves in this direction came from some quite ‘enjoyable’ experiments performed by Plautz and Kay in Drosophila in 1997 [6]. Transgenic Drosophila was produced, in which a reporter gene construct consisting of the period gene promoter, driving the expression of luciferase, was stably inserted into the fly genome. When this gene was naturally activated or ‘turned-on’, the flies would glow. What Plautz then did, possibly in a fit of ‘schoolboy angst’, was to pull off the flies’ wings, legs and antennae and place these into a luminometer. The interesting, if somewhat unexpected, result was that expression of the period gene continued to be rhythmic, and this oscillation could be entrained by exposure to a light–dark cycle. So these Drosophila tissues must contain directly light-responsive circadian clocks.

It took a further year, however, before similar evidence of decentralized circadian pacemakers in vertebrates was produced. The first of these stories showed the existence of clocks within a variety of vertebrate cell lines [7]. If cells, such as Rat1 fibroblasts, are initially serum-starved and then serum-shock-treated, several days of circadian oscillation can be seen in the transcription of many central clock genes. The issue of whether these serum treatments simply start this oscillation or synchronize already oscillating cells was more recently answered by using single-cell imaging approaches by Schibler and co-workers [8]. In the same year, tissues, such as heart and kidney, were dissected from adult zebrafish and placed into culture for up to 5 days [9]. Time points were then collected across the circadian cycle, and using RNase protection assays to measure the amount of mRNA, a clear rhythm in the expression of the gene clock was revealed. Expression of clock peaked at dusk, in the early evening, the same time as seen in these tissues in vivo. A self-sustaining circadian pacemaker was clearly present in hearts and kidneys even after several days in culture. These studies were subsequently extended to show that the majority, if not all, of tissues within the fish body contained their own circadian clocks. This observation raised the obvious question of how these peripheral clocks become set or entrained to the natural

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Abbreviations used: BrdUrd, bromodeoxyuridine; SCN, suprachiasmatic nucleus; TMT, teleost multiple tissue
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Figure 1 | Luminescent measurements from two wells of a 96-well plate, containing zebrafish PAC2 cells transfected with the reporter construct, period1-luciferase
These cells show a robust oscillation in period gene expression, with a peak in the early day. The rhythm persists but dampens down when the cells are placed into constant darkness. However, a robust oscillation is restored upon return to a light–dark cycle.

environmental light–dark cycle. The ‘smart money’ was on either a humoral signal, such as melatonin or corticosterone, or a series of neural pathways, linking a central clock to the periphery. The slightly more outrageous possibility was along the lines of the biology of Drosophila, with directly light-responsive organs and tissues. This direct, and rather simple, method of entrainment turns out to be the correct answer [10]. A number of hearts were dissected from zebrafish on a typical light–dark cycle, lights on at 09:00 h in the morning. One-half of these hearts was placed into an incubator, illuminated by a fibre optic light source on the same light–dark cycle as the adult fish. The other half of the hearts was placed on to an inverted light–dark cycle, equivalent to flying those hearts to Australia. The clocks within this second batch of hearts rapidly re-entrained to the new light cycle, within 1 day of being placed under these conditions. These hearts not only contained a clock, but could also detect light, the signal required to set the clock. This was true for most of the organs in the body, and so tissues as diverse as heart, muscles, gills and even the whole blood could directly detect light in the environment. In this particular aspect, zebrafish and Drosophila appeared to be quite similar [11]. More recently, it has been confirmed that mammals also contain independent circadian pacemakers within their tissues [12]. These clocks are robustly self-sustaining, but are not themselves directly light-responsive, as in the fish. The eyes are still required for entrainment of these peripheral mammalian pacemakers, at least to the environmental light–dark cycle.

As with certain mammalian cell lines, we found that cells produced from zebrafish embryos also contain circadian clocks [10]. However, unlike their mammalian counterparts, they do not require any serum pretreatments in order to show these oscillations. Instead, all that is required is to place these cells in their culture flasks on to a light–dark cycle; as with the adult tissues, these cells are directly light-responsive. In fact, to date, having made numerous zebrafish cell lines, we have yet to generate one that does not possess this direct light responsiveness. So, it appears that not only is the core circadian clock mechanism contained within a single cell, but also the light sensing machinery, photopigments and signal-transduction cascades. In order to improve our analysis of this cell system, it was clear that we needed not only to automate these assays of rhythmicity, but also to increase the level of sensitivity. To do this, we employed the same approach as that of Kay and co-workers [6], by creating luminescent cell lines containing clock promoter constructs driving the expression of the firefly luciferase gene. The initial cell lines produced contained the zebrafish homologue of the mammalian period1 gene and showed robust, high-amplitude oscillations, with peak expression 3 h after lights-on (see Figure 1) [13]. The rhythm dampens down after a number of days in constant darkness, but is rapidly restored upon returning the cells to a light–dark cycle. Although we believed that each cell was a complete and independent light-responsive, circadian clock system, this was not yet absolutely confirmed. Furthermore, the question remained as to whether light was acting simply as an entraining signal, synchronizing a population of oscillating but out-of-phase cells, or whether the action of light on constant dark-maintained cells was to actually start the clock mechanism itself oscillating.
Figure 2 | Single-cell luminescent imaging of zebrafish cells transfected with the reporter gene construct, $\text{period1-luciferase}$

In this experiment, cells were maintained in constant darkness for several months. Nevertheless, oscillations can be recorded in single cells, but the phase of these oscillations is random. The circular statistical plot demonstrates this random distribution of phase. Single cells do possess a free-running clock that is maintained after long periods in the dark. Modified from [14].

To address this question, Carr and Whitmore [14] initially generated clonal cell populations of zebrafish luminescent reporter cells using FACS. Individual cells were each placed into one well of a 96-well plate and grown to confluence. Each clonal line could then be directly checked for rhythmic gene expression, as well as the level of luminescence at peak expression. The brighter cell lines were then selected for further examination employing luminescent imaging using a highly sensitive photon counting camera. For the initial experiments, cells were maintained in constant darkness for several months. When examined at the population level on a luminometer, such cells showed no circadian oscillations, meaning either that the clock had stopped or that all of the cells had drifted completely out of phase with each other. These cells were then placed on an inverted microscope with the imaging camera attached for 3 days of continuous photon counting in the dark. All cell handling, media changes, cell splitting etc. had to be performed in complete darkness, as we found that any exposure to light during this handling would establish an oscillation within the population. Our initial results showed that cells maintained for a long period in the dark did still continue to show circadian oscillations in gene expression at the single-cell level (see Figure 2). Light is not necessary to start the clock, which can run for months, if not years, in culture.

In a second series of experiments, this constant dark population of cells was given a single 15 min pulse of light during the imaging/photon counting experiment [14]. The results were quite striking in that the whole population of individual luminescent cells phase-shifted in response to this light pulse, and in fact, continued to oscillate from a common phase in the circadian cycle following this treatment (see Figure 3). In other words, this 15 min light pulse ‘moved’ the clock within these individual cells to a common time-of-day. This time or phase, interestingly, corresponds to the early morning, just a few hours after the normal ‘lights on’ signal. Extrapolating from these cell data to the tissues and organs of the whole animal, we believe that the light experienced by cells within tissues of the body at dawn strongly resets these peripheral clocks. These body clocks are rapidly shifted to a common phase, which happens to be the equivalent to the dawn point of the circadian cycle. For this to occur, these zebrafish cells must have very strong resetting responses to short light pulses, and this is indeed the case, as the circadian clock in zebrafish possesses a very high-amplitude phase response curve, i.e. the oscillator can be shifted in phase by many hours following a short light stimulus [13]. Clearly, the cells within the zebrafish body are strongly light-responsive in terms of their circadian characteristics. Rather interestingly, when the circadian clock oscillation is measured within these luminescent cell populations, the range, or spread, of free-running period was much greater than expected. In fact, as we were working with clonal, genetically identical cells, our expectation was that individual cells would run with almost identical periods, very close to 25 h. This was far from the case, with cells drifting out-of-phase after only a few days in constant darkness – a consequence of this wide range of free-running periods. It is interesting to speculate that, as these cells are strongly light-responsive, the requirement for a tightly regulated free-running period is significantly lowered. In such a system, natural selection has ‘worked’ on the light-input pathway, and in the absence of any strong selective pressure, the mechanism responsible for generating the clock period has become rather ‘sloppy’ or imprecise.

All of this leads to the obvious question, what is or are the photopigments responsible for this cell–tissue light
response? Well, unfortunately, we do not yet know absolutely. However, a number of possible candidates do exist, and these are currently being tested using these cell lines. The currently ‘hot’ circadian photopigment molecule in the mammals, melanopsin, is not likely to play a role in this process [15]. Expression of melanopsin in zebrafish appears to be restricted to a subset of retinal horizontal cells, and is barely detectable within the pineal gland, never mind the other tissues of the body from which it appears to be absent. One unusual opsin, however, called TMT (teleost multiple tissue) opsin, is a potential candidate for the circadian photopigment, since it is expressed widely in a range of tissues, including the light-responsive PAC2 cell lines [16]. At this time, TMT opsin is the only photopigment identified in these cell lines; although this may be a compelling correlation, there is at this time no functional data proving that TMT plays a central role in clock entrainment in zebrafish. Cryptochromes have been suggested as a likely candidate for the circadian photopigment in fish [17]. They have been strongly implicated in this role in Drosophila, and certainly fulfill the wide tissue expression patterns one would require of a circadian photopigment in the teleosts [18]. There are at least six distinct cryptochromes in zebrafish, but to date, again, there is no functional data supporting a role for this family of molecules in clock entrainment [19]. In fact, a stronger case can be made for them fulfilling a central role in the clock mechanism and possibly in the light entrainment pathway itself (T.K. Tamai and D. Whitmore, unpublished work). With the lack of a clear, proven photopigment in these tissues and cells, the only reasonable strategy forward is to perform a large-scale mutant screen for molecules involved in the light-input pathway in zebrafish. Such a screen is currently being performed at the Max-Planck Institute for Developmental Biology in Tübingen, Germany. This approach, although a long-term effort, is likely to provide many answers to this unusual signalling process, and may lead to the identification of the circadian photopigment in zebrafish, that is as long as no multiple, redundant photopigment molecules are involved.

Another issue of considerable interest is, why is this cellular light responsiveness so widespread amongst tissues and cells? A possible hint came from an examination of light-dependent changes in gene expression in early zebrafish embryos [20]. Zebrafish lay their eggs usually approx. 1 h after lights on at dawn, and become transcriptionally active,
i.e. the zygotic genome ‘turns on’, at the mid-blastula transition, approx. 5 h after fertilization. Tamai et al. [20] showed that light pulses given to these early embryonic stages increased the transcription of the period2 gene, as early as the beginning stages of gastrulation, very shortly after the mid-blastula transition. This light responsiveness occurs well before the differentiation of any neuronal or classical light-responsive structures in the embryo. However, not only was the period2 gene induced, but also transcription of 6-4 photolyase, a protein involved in DNA repair. Control embryos raised in the dark lacked detectable expression of this gene. The importance of this early embryonic light detection was clearly demonstrated when a batch of sibling embryos was divided equally and one-half was raised on a normal light–dark cycle, with the others being maintained in constant darkness. At a selected time point during day 1 of development, both groups received a short pulse of UV light and then were returned to their respective lighting regimes. On the following day, it was found that, on an average, 80% of the dark-raised embryos had died as a consequence of this UV exposure, whereas only 20% of their siblings, raised in the light, had died [20]. Clearly, this early embryonic light sensitivity can play a critical role in providing protection against environmental stressing agents, such as UV light. It is interesting to speculate, therefore, that perhaps this widespread cellular light sensitivity, in terms of circadian clock entrainment, has some evolutionary link, and probably even a mechanistic link, to the processes involved in the activation of DNA repair.

Additional information that tends to support this idea, and also possibly explains the importance of a circadian clock during early embryonic development, stems from the observation that the cell cycle in young zebrafish larvae appears to be clock-regulated. Using BrdUrd (bromodeoxyuridine) incorporation as a measure of the timing of DNA replication, Dekens et al. [21] were able to show that, in the case of embryos entrained to a light–dark cycle, DNA replication is restricted to the late day/early evening. In contrast, sibling embryos raised in constant darkness showed an even level of BrdUrd incorporation, or DNA replication, across the circadian cycle. It seems likely, therefore, that one role of the early embryonic circadian clock is to regulate and restrict DNA replication, and possibly consequently mitosis, to the night or dark region of the circadian cycle. In this way, the amount of UV exposure received by cells undergoing these highly sensitive cellular events may be reduced.

In the future, it will be interesting not only to explore the cellular signalling events that underlie this general light responsiveness and the regulation of events like cell cycle and DNA repair, but also to determine the breadth of additional cellular events that are altered by this general cellular light sensitivity.

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