Short-term culturing of teleost crystalline lenses combined with high-resolution optical measurements

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Abstract Culturing whole lenses is a frequently used method for studying regulatory events on the lens in controlled environments. The evaluation methods used often fall under two categories, molecular or optical. The main benefit from optical measurements is that they directly detect changes in the lens' main function, i.e. refracting light. However, these measurements often have rather low resolution or yield results open for subjective interpretation. Here we present a short-term crystalline lens culturing technique combined with a high-resolution optical measuring method. There are two main advantages of using teleost lenses compared to mammalian lenses. Teleost tissue generally has a higher tolerance than mammalian tissue with regard to temperature and nutrient fluctuations. Teleost lenses are structurally more robust and can be excised from the eye without disturbing form or function. The technique is developed for short-term culturing (3 h), however, the lenses appear viable for at least 24 h and longer culturing may be possible. The technique is resistant to small variations in osmolarity and yields quantitative datasets for further analyses and statistical treatment.

Keywords Lens · Organ culture · Teleost · Optical measurements

Introduction

Organ cultures using whole crystalline lenses are commonly used to study lens physiology and lens related problems, such as cataract, in a variety of vertebrates, from teleosts to primates (Hikida and Iwata 1986; Kamiya and Zigler 1996; Zhou and Menko 2002). There are two main types of screening method used in association with lens culture systems, molecular assays and optical evaluation. With optical evaluation, the function of the lens is directly assessed which is an advantage when investigating for instance cataractogenesis. Since optical evaluation may be non-invasive with regard to the lens, repeated measurements can be done over time. However, results from such methods are often subjective or have relatively low resolution. Direct photography and subsequent image analysis for determination of affected areas have previously been used (Zhou and Menko 2004). Other methods are for instance those used by Hightower and Dering where the opacity of the lens was measured by determining the reduction in light intensity after the light beam had passed through the lens (Hightower and Dering 1984), and the optical method for studying the quality

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of the lens during culturing (Sivak et al. 1986). The latter method, although yielding results with high-resolution, predominantly measures the center of the lens, leaving changes occurring in the lens periphery unaddressed.

Vertebrate lenses have an internal refractive index gradient that improves image quality. The bulk of the lens consists of many layers of thin lens fiber cells that have broken down all of their organelles, including the nucleus, which increases lens transparency and further improves image quality. New cells arise continuously in a germination zone close to the lens surface and the lens continues to grow throughout life. Since the index gradient has to be maintained during growth, lens cells have to adjust refractive index of the cytoplasm despite having no organelles. This is a problem shared by all vertebrates, from lampreys, the most basal group of vertebrates, to humans. It is therefore likely that the regulatory mechanisms controlling the optical properties of the lens have evolved very early and that the basic mechanisms are similar in all vertebrates, including teleosts. Teleost tissue is generally less sensitive than mammalian tissue with regard to nutrients and temperature fluctuations. It can therefore be cultured using a basal medium at room temperature, factors that reduce the risk of bacterial growth and tissue deterioration. Teleost lenses are physically robust because of their spherical shape and high internal pressure, such that they remain structurally unchanged when removed from the eye. Furthermore, the lens is the only refractive element in the teleost eye, in contrast to terrestrial animals in which the cornea is also optically important. Measuring the optical properties of teleost lenses therefore returns information that is directly relevant for the properties for the image formed on the retina.

Another favorable feature of fish visual systems is that the retina dark-adapts by physically moving the cones out of the focal plane of the lens and the rods into the focal plane. This re-organization, called *retinomotor movements*, gives the animal an all cone retina during daytime and an all rod retina at night, and takes about an hour (Burnside 2001). We have shown in the South American cichlid Blue Acara, *Aequidens pulcher*, that the optical properties of the crystalline lens change from day to night and that the dark-adapted lens appears to better serve a dark-adapted retina. The change is located mainly in the

outermost 40 percent of the lens radius (Schartau et al. 2009). To identify the mechanisms behind this change, an in vitro testing system is essential. Detecting these changes requires a measuring method with high resolution that provides information over the entire diameter of the lens.

A chemically undefined medium, although most similar to biological fluids, has major shortcomings—unknown composition and batch variation being the major ones. The media used in lens culturing often contain bovine serum or similar additives, which can make interpretation of the results more difficult. An observed effect may have been caused by the test substance or by variations in unknown components in the additive. Using a defined medium giving complete control over its composition excludes such problems at the expense of the viability of the lens. However, when studying changes occurring daily within the animal, a few hours of culturing should be sufficient to identify the mechanisms.

Our goal of was hence to combine several methods into one technique where lenses could be cultivated for at least 3 h in a basal defined medium and studied optically without being handled or moved. Small changes had to be observable in the whole lens, especially the periphery, and the culturing chamber had to be designed so that it accommodated for all requirements of the method of measurement. High throughput by simultaneous handling of several lenses was another important aim. Here we describe an experimental set up that fulfills these requirements using existing measurement methods (Malkki and Kröger 2005; Schartau et al. 2009).

Material and procedures

Tissue

All teleost lenses with optical radial symmetry can be examined using this technique. In the work presented here we used lenses from *Aequidens pulcher*. The animals were sacrificed by pithing and the lenses, approximately 2–3 mm in diameter, were aseptically extracted as previously described (Malkki and Kröger 2005). The posterior end of the eye was removed and all but one of the ligaments suspending the lens in the eye (Khorramshahi et al. 2008) was severed close to the lens. One ligament was spared serving as a handle

such that the lens could be transferred to the culture chamber without touching it since any direct handling of the lens would damage the lens surface. The vitreous body contains no cells and accidental transfer of a small part poses no risk to the experiment. Any active mediators transferred would be significantly diluted in the large volume of the culturing chamber and the vitreous humor is optically identical to the medium.

Culturing conditions

For our purposes a medium modified from H10 was used. Phosphate-free media, such as H10, are frequently used in teleost tissue culturing but also for mammalian lens cultures (Hikida and Iwata 1986; Laycock et al. 2000; Takahashi and Copenhagen 1992; Young et al. 2000). The technique sets no technical limitations for the medium and H10 can easily be replaced by a more complex medium if the experiment requires it. However, one should be careful with additives, such as bovine serum albumin, BSA, that might cause film coating since this might disturb the optical measurements. The H10 composition was 120 mM NaCl, 2.50 mM KCl, 0.80 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose and 3 mM HEPES. The pH was adjusted to 7.3 and osmolality measured to 280 mOsm (3300 Advanced Micro Osmometer, Advanced Instruments Inc.). To determine the osmolality in the eye, the vitreous bodies from 2–3 fish were pooled and centrifuged for 30 min at 8,000 rpm. The osmolality of the extracellular liquid was then measured. The osmolality of the medium was adjusted without altering the Na^+/K^+ ratio. Since oxygen tension is low close to the lens, the culturing medium was degassed by freezing and thawing prior to use. All experiments were conducted at room temperature, 20–22 °C.

Culturing chambers

The culturing chambers had an approximate volume of 10 mL. Three of the sides were transparent while the fourth side and the bottom were black. To allow for optical measurements, the chambers also needed two flat surfaces perpendicular to each other, one for light entry and the other for observations (see *laser scanning*). In the center of each chamber there was a rotational stage with a lens holder. The holder

elevated the lens from the bottom of the chamber, thus minimizing glare and optical distortions during measurements. These requirements disqualified most of the commercial available culture chambers. The chambers were custom-built using acrylic glass for the main body and a microscopy cover slip as entrance window for light. Four identical culturing chambers were mounted on a motorized linear stage to allow for quadruple samples (Fig. 1).

Laser scanning

Laser scanning is commonly used to determine the optical properties of teleost lenses (Kröger et al. 1994; Malkki and Kröger 2005; Sivak et al. 1986). Four chambers were mounted on a motorized linear stage (Linios x.act LT 200-2 ST) so that each chamber could be moved into the measuring position. In position, white light (Argon arc lamp) or a 543.5 nm laser beam was directed in through the front window of the chamber. A microscope (Olympus U-TR30-2) and a digital video camera (Sony HDR-HC7E, hdv) were exchangeably mounted above the measuring position. White light and the microscope were used to identify the optical axis of the lens so that the lens could be rotated into the correct orientation (Fig. 1b). The microscope was then swiveled away to make room for the video camera. The laser beam was scanned in the plane of the optical axis of the lens while the video camera recorded the scan. Each lens was scanned twice within less than one minute. Visualizing the laser beam in the culture chamber required the addition of 1 μL 0.1 μm polystyrene beads (Sigma Aldrich) for every 10 mL of medium.

Data processing has previously been described in detail (Malkki and Kröger 2005). In short, the longitudinal spherical aberration (LSA) of the lens was determined from the video recordings of the scanning (Fig. 1). The LSA curves were composed of 200 data points across the lens diameter and quantitatively described the variation in refractive power across the aperture of the lens. The average of both scans was used as one data point (Fig. 2). The variation between scans performed on the same lens and variation between the left and the right lens from the same animal were smaller than the variation between animals. The effect of a treatment can therefore be evaluated by using one of the animal's lenses as an internal control, which reduces the

Fig. 1 Culturing and measuring set-up. **a** The set-up was assembled around two motorized linear stages. The first stage held the four culturing chambers and moved the chambers into the measuring position. In position, light could be directed into the chamber through the front window that was slightly oblique (3 degrees) to avoid internal reflections leading to ghost images. A digital video camera and a microscope were exchangeably mounted above the lens in the measuring position (marked with a *shaded box*). The second stage held a mirror and a focusing lens that reduced beam width. The laser beam was scanned through the lens in the chamber by moving this stage back and forth. **b** The lenses were placed on a rotational holder (not shown) in the center of each chamber and orientated so that the optical axis of the lens was parallel to the

laser beam (picture not drawn to scale). The recorded video was exported to single frames and *beam entrance position* (BEP) and *back center distance* (BCD) were determined for each frame. The BEP is the lateral distance from the optical axis of the lens to the laser beam, while BCD is the longitudinal distance from the center of the lens to where the laser beam intercepts the optical axis. Both distances are expressed in units of lens radius (R). BCD as a function of BEP gives a *longitudinal spherical aberration* (LSA) curve. **c** Representative LSA curve describing the variation in refractive power over the radius of the lens. Higher BCD values indicate lower refractive power (longer focal lengths) while lower BCD values indicate higher refractive power (shorter focal lengths)

masking effects of individual variations (Fig. 4). Changes in LSA were monitored over time by subtracting the LSA curve at 0 h, the control, from later recordings. This results in a differential LSA curve that describes the changes that occur over time in each lens (Fig. 3).

Visual inspection

Visual inspection was conducted by photographing a grid through the fish lens every 24 h until the grid was clearly distorted. The lens was kept in a Petri dish filled with modified H10 medium (280 mOsm). The

Fig. 2 Results from visual inspection and laser scanning. One lens from each animal was measured by laser scanning while the other was placed in a Petri dish and used for visual inspection. The changes seen by visual inspection were subtle at first. At 72 h the visualized grid appeared slightly distorted and it was clearly distorted after 96 h. The lines appear blurred and curved in the periphery of the lens. The LSA curves (*lower panel*) deviated from the initial measurement already after 24 h. The lens radius remained constant throughout the experiment. Scale bar 1 mm

Fig. 3 Effects of osmolarity. Lenses were excised and scanned at 0 and 3 h in different osmolarities. The differential LSA curves were obtained by subtracting the LSA at 0 h from the LSA at 3 h. Osmolarities below 330 mOsm decreased the refractive power in the periphery while higher osmolarities increased it. The changes occurring at osmolarities between 315 and 345 mOsm were small. It is therefore likely that minor deviations in osmolarity from 330 mOsm have little effect on the optical properties of the lens

images were taken with a digital camera (Sony, DSC-F707) through a microscope (Zeiss Stemi SV 6).

Viability test

Lenses were excised and placed in an osmolarity-adjusted medium containing 0.1% Lucifer Yellow (LY dilithium, Sigma Aldrich). Images were taken using a confocal microscope (Zeiss, LSM 510 Meta) in the mid vertical plane of the lenses every 24 h until LY had entered the lens fiber cells.

Results and discussion

Visual inspection and laser scanning were tested against each other using the original medium osmolarity (280 mOsm) that has previously been used for experiments on teleosts eyes described in the literature. One lens from each animal was used for either laser scanning or visual inspection. Scanning results from the left and right lenses from the same animal are almost identical (Fig. 4), such that one lens (untreated) can be used as a control for the other (treated).

The grid visualized through the lens was clear and undistorted for the first 48 h, (Fig. 2). After 72 h the image seen through the lens became blurred in the periphery of the lens and after 96 h the lines making up the grid were clearly distorted. With laser scanning, an optical change was detectable already after 24 h. The optical properties deteriorated further from 48 to 96 h. Laser scanning revealed changes earlier than visual inspection and provided a quantitative measure for the changes occurring in the lens (Fig. 2).

The osmolarity measurements of the vitreous yielded an average of 330 mOsm. The lenses where cultured for the targeted duration, 3 h, using different osmolarities to test the robustness of the method. The optical changes that occurred were highly dependent on osmolarity. The scanning showed that an osmolarity lower than 330 mOsm decreased the refractive power of the lens periphery while a higher osmolarity had the opposite effect. The optical properties of the lens remained essentially unchanged when the osmolarity of the medium was kept between 315 and 345 mOsm, indicating that there is an acceptable

Fig. 4 Optical variation between scans and lenses. The variations between the scanning results from each animal were small which is indicated by the similarity of the curves. Repeated scans on the same lens reveal only minor differences, confirming that laser scanning is a robust method. The similarity between *right* and *left* lens indicates that one lens can be used as an internal control for the other lens. After 3 h in the culturing medium (330 mOsm) there was only a small change in the optical properties, however, the change was

identical between lenses from the same animal indicating that the initial scan can be used as an internal control. There was a difference in optical properties between the lenses from different animals and one should be mindful when using results from one animal as a control for results from another individual. The method has poor resolution at the most central values (<0.05 BEP) and the values have therefore been removed

interval in osmolarity in which the method can be used (Fig. 3). It is likely that the lower osmolarities resulted in water diffusing into the lens, thus diluting the proteins in the outermost cell layers and reducing the refractive power in the lens periphery. The higher osmolarities probably increased the refractive power in the periphery due to water diffusing out of the lens, thus concentrating the proteins in these cell layers.

The dye, LY, does not penetrate intact cell membranes but passes from cell to cell through gap junctions (Stewart 1981). Damaged or dead cells would result in the dye permeating into the cells and spreading within and between the lens fiber cells. Confocal microscopy images showed that the lens remained free of LY after 24 h of incubation in an

osmolarity-adjusted medium (330 mOsm). However, 48 h after initiation of the experiment the lens was completely stained (Fig. 5). This shows that the lens fiber cell membrane integrity was maintained for at least 24 h.

Using several chambers simultaneously not only benefits the general throughput of the method but also allows for comparing the effect of a mediator or condition on one lens while using the other lens of the same animal as control. Treating the lens from one eye while using the other as internal control reduces the effects of individual variation on the results, thus minimizing the number of animals needed. The development of the optical properties over time could be followed by using the first data

Fig. 5 Lens viability. The lenses were placed in a 330 mOsm medium containing 0.1% Lucifer Yellow and images were taken in the mid horizontal plane. The lens fiber cells remained unstained for at least 24 h. At 48 h the whole lens was almost uniformly stained by the dye and distinguishing the lens from

the background had become difficult. To visualize the lens edge, the laser intensity had to be decreased compared to that used for the earlier images, which led to noisy images. Scale bar 0.1 mm

point as a control for the subsequent measurements (Fig. 4). It should be mentioned that there are no technical limiting factors that prevent the number of culturing chambers to be increased beyond four. Experiments extending beyond 24 h might be possible with additives, e.g. glutamate or serum, and although aseptic dissection and low culturing temperature reduce the problem of bacterial growth, longer culturing periods might also require antibacterial additives. The use of serum would, however, reintroduce the problem of having unknown compounds in the culturing medium. It is furthermore important to consider that certain additives, like BSA or complete serum, might cause film coatings and interfere with the optical measurements.

Conclusion

Lenses from two fishes can simultaneously be cultivated and measured in single chambers containing a basal medium. The technique measures optical changes across the entire diameter of the lens with higher resolution than other methods used. The technique is robust for changes in osmolarity and small deviations do not affect the results. The lenses remain optically unchanged for more than 3 h and are viable for at least 24 h.

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