

# Structural changes in the crystalline lens as a function of the postmortem interval assessed with two-photon imaging microscopy

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**Abstract:** The properties and structure of the crystalline lens change as time after death passes. Some experiments have suggested that these might be used to estimate the postmortem interval (PMI). In this study, the organization and texture of the rabbit lens were objectively evaluated as a function of the PMI using two-photon excitation fluorescence (TPEF) imaging microscopy. Between 24 h and 72 h, the lens presented a highly organized structure, although the fiber delineation was progressively vanishing. At 96 h, this turned into a homogeneous pattern where fibers were hardly observed. This behaviour was similar for parameters providing information on tissue texture. On the other hand, the fiber density of the lens is linearly reduced with the PMI. On average, density at 24 h was approximately two-fold when compared to 96 h after death. The present results show that TPEF microscopy combined with different quantitative tools can be used to objectively monitor temporal changes in the lens fiber organization after death. This might help to estimate the PMI, which is one of the most complex problems in forensic science.

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#### 1. Introduction

Microscopy imaging techniques are of great importance in life sciences. In particular, two-photon excitation fluorescence (TPEF) is a powerful multiphoton microscopy modality used to evaluate the structure and properties of biological tissues under different conditions. Since its first application more than 30 years ago [1], it has successfully transitioned from its invention to a useful imaging method covering a broad range of fields in biomedicine [2]. This is based on the quasi-simultaneously absorption of two infrared photons to emit only one visible photon. Given the endogenous nature of the signal, the use of markers is not necessary. Furthermore, it provides high optical sectioning capabilities, and allows enhanced depth penetration and reduced phototoxicity [3].

Visualization and analyses of ocular tissues have greatly benefit from this imaging approach. In particular, the spatial distribution of different retinal and corneal cells has been studied with TPEF microscopy [4–11]. Moreover, TPEF images have also provided information to differentiate normal from abnormal ocular tissues (as a result of disease, age or pharmacological treatments) [12–16].

All these studies were centred on the cornea and the retina, and to the best of our knowledge, the crystalline lens has been hardly explored through TPEF imaging [17]. Recently, Paidi and collaborators successfully reported a non-invasive study of the cell organization in the mouse crystalline lens combining adaptive optics and TPEF microscopy [18]. This ocular component is responsible for the accommodation, that is, the ability of the eye to provide clear vision at

different distances by modifying the refractive power (i.e. the shape of the anterior and posterior surfaces) [19]. Apart from TPEF microscopy, other methods are used to visualize the fibers of the crystalline lens, including conventional widefield optical microscopy and scanning electron microscopy [18,20].

The lens suffers life-long optical and physical changes (refractive index, biomechanics, curvature, transparency) which have been object of study for many years [19]. Moreover, it has been also reported that changes after death might be useful to estimate the postmortem interval (PMI) [21,22], defined as the elapsed time since the moment of the death. However, these modifications suffered by the lens were mainly studied from a macroscopic point of view and an in-depth microscopic analysis is lacking in the literature.

The estimation of the PMI within the context of a medicolegal death investigation is simultaneously one of the most common and most difficult questions to answer in forensic science [23]. Numerous methods have been proposed over the years to be used in both short and long timeframes. *Algor mortis*, or body cooling, is one of the most frequent procedures for early PMI estimation. However, corrections accounted for weigh or the conditions of the body before and after the death need to be included in the temperature-based model [24]. Due to the complications of conducting experiments on human donor bodies and the impossibility of finding a high enough number of retrospective cases with similar intrinsic and contextual conditions, much of the research done on body decomposition has been done using animal models [25].

Methods based on analyses of vitreous humour biochemical changes (such as potassium  $[K^+]$  concentration) have become of interest over the past years [24]. For longer timeframes, PMI estimation is based on methods such as forensic entomology, tissue degradation analysis or morphological changes [26]. For most of these methods, the main problem of determining the PMI is common: the accuracy of the assessment is reduced as the PMI increases due to the variety of intrinsic and extrinsic factors that can affect the corpse [27]. Unfortunately, nowadays there is still not consensus about the best technique to accurately assess the PMI.

The time of death has particular relevance in criminal scenes since an incorrect PMI determination might lead to serious miscarriages of justice. An appropriate and accurate PMI assessment should be based on objective, reproducible, reviewable and critical measures. Then, it's important to reduce subjective judgements, and measurements must be mainly based on scientific evidence.

In this sense, herein we have used TPEF imaging microscopy to investigate the alterations in the crystalline lens as a function of the PMI. Several algorithms have been developed to analyze texture and assess structure-related parameters.

#### 2. Methods

#### 2.1. Samples

Crystalline lenses from 8 rabbits were used for the purpose of this experiment. The animals were provided by a local slaughterhouse and killed for human consumption. Just after sacrifice, the eyes were enucleated and moved to the lab. There, the ocular globes were left exposed to the air at a temperature of 21°C during 24, 48, 72 and 96 hours. At those postmortem time points each group of lenses (N = 4 for each one) were isolated from the eye by making a lateral incision and carefully cutting the ciliary body. Once extracted, each lens was washed in physiological saline solution, fixed in 10% neutral-buffered formalin and embedded in paraffin. Tissue sections of 4- $\mu$ m in thickness were obtained from each paraffin block and mounted on a microscope glass slide. All the samples were left unstained for TPEF imaging.

The entire experiment followed the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Ethical Committee of the University of Murcia (ID-3114/2020).

# 2.2. Experimental setup

The setup used to image the rabbit lenses was a previously described multiphoton microscope [6]. A sketch of the instrument is depicted in Fig. 1. An 800-nm femtosecond (fs) laser (76 MHz repetition rate) was used as illumination source. The average laser power at specimen was 60 mW and it was controlled by a neutral density filter (NDF). The beam is guided towards the XY scanning unit and enters an inverted commercial microscope using additional optics. It is then reflected by a dichroic mirror (DIC) towards a dry objective (20x, NA = 0.5). The TPEF signal generated was collected in the back-scattering geometry by the same microscope objective. A spectral long-pass filter (TPEF filter, 435-700 nm) located in front of the detection unit was used to isolate the TPEF signal. A photomultiplier tube (PMT) was used as a detector. Image acquisition was controlled by a custom software in LabView.



Fig. 1. Schematic of the experimental TPEF microscope. See text for details.

### 2.3. Image analysis

For each specimen 3 randomly selected locations were imaged and for each location 3 TPEF images were acquired. Every set of 3 images was averaged to reduce noise. Image processing was applied to this final averaged image. Individual frames were recorded at approximately 1 Hz. All imaged areas were  $180 \times 180 \,\mu\text{m}^2$  in size  $(256 \times 256 \,\text{px}^2)$ . Several algorithms and parameters were used to extract objective information from the lens structure. These included spatial organization, texture analysis and fiber density. An extended explanation of these image analysis procedures can be found below. The statistical analysis was performed with the open-source software jamovi.

### 2.4. Structure tensor

To explore the arrangement of the crystalline lens features, the structure tensor has been used. This procedure was extensively described in [28]. In brief, this is a mathematical tool providing quantitative information on both the degree of structural organization and the preferential orientation (PO) of spatially resolved structures. It is based on the calculation of the partial derivatives along the X and Y directions of the image (i.e., gradient direction and magnitude). Once the eigenvalues of the partial derivative matrix are known, it is possible to calculate the angle associated to the PO ( $\theta_{PO}$ , if any) and the structural dispersion (SD). SD is defined as the standard deviation of the histogram of orientations. As a rough idea, the higher SD, the lower the

alignment of the fibers along a PO. In particular, if  $SD \le 20^\circ$ , the fibers present a quasi-aligned distribution. When,  $SD > 40^\circ$ , the image contains a non-organized arrangement. The condition in between is associated to a partially organized disposition [28]. This software was also developed in Matlab.

Figure 2 depicts illustrative examples of images from ocular tissues with the corresponding histograms of PO. In particular, the SD values of these images are 19° and 42°, which correspond respectively to fairly organized and disorganized arrangements.



**Fig. 2.** Examples of the use of the structure tensor in two biological images (left panels) presenting different structural organizations. Plots on the right are the corresponding PO histograms. These represent the frequency of appearance of a certain orientation of the sample's features within the image. The values of SD are inserted for clarity.

# 2.5. Gray level co-occurrence matrix (GLCM)

In 1973, Haralick proposed a method to classify images based on their texture by performing a statistical analysis of the pixel intensity values across the images. This was called the Gray Level Co-occurrence Matrix (GLCM) [29]. This procedure (also referred as Co-occurrence Distribution) computes how often a pixel with gray level *i* occurs either vertical, horizontal or diagonally to adjacent pixels with value *j*. In other words, the GLCM measures the frequency at which a specific spatial combination (distance and angle) of two pixels with intensity values, *i* and *j*, appears in the image. This procedure is used to calculate different statistical parameters that provide information on the spatial distribution of the features within an image. Herein we have used two parameters computed from the GLCM: homogeneity and energy.

Homogeneity measures the uniformity of the image. It provides larger values for smaller gray level differences in pair elements. It ranges between 0 and 1, reaching the maximum when all pixels in the image have the same value. Energy (also known as uniformity or angular second moment) determines if the presence of a certain gray level in the image is dominant. It detects disorders in textures and its range is also [0,1]. Higher energy values occur when the gray level distribution is fairly constant. In general terms, energy and homogeneity decrease with increasing image quality. Moreover, it is interesting to note that if the contrast of an image increases, homogeneity reduces, while energy keeps constant.

This tool has been previously used in biomedical imaging analyses and it has become a useful technique to study healthy and pathological tissues [30]. Since MatLab includes a specific toolbox for GLCM, this was easily implemented in our image analysis script.

#### 2.6. Fiber density

A custom software was specifically developed in Matlab to measure both the size and the density of the lens fibers appearing in the TPEF images. For this goal, each original TPEF image (see Fig. 3(a)) was processed in two steps. First, the image was filtered by means of a Canny filter [31] (Fig. 3(b)). This allows detecting the edges of the fibers within the original image, what avoids artifacts when computing the PO (i.e. the angle  $\theta_{PO}$ ) of the tissue structures.



**Fig. 3.** Representative example of the described procedure. (a) TPEF image; (b) Canny filtered image; (c) adaptive thresholded (and binarized) image.

For the second step, an adaptive threshold procedure was applied to the original grayscale image to increase the contrast of the fiber content (Fig. 3(c)). The procedure is based on the method by Bradley & Roth, described in [32]. This uses a sensitive parameter, *t*, that ranges between 0 and 1. If the gray level of the actual pixel is *t* times less than the local mean intensity, then the pixel value is set to 0. If, on the other hand, it is greater, then the pixel value is set to 255. For our experiment *t* = 0.05 was used. From this thresholded image, the correlation parameter was computed through the GLCM. This operates as follows. As explained above, GLCM computes how often pairs of pixels with a specific value and offset occur in the image. Then, the distance and the angle between pairs of pixels of interest are required as an offset. In practice, this is a 2-element vector that sets the specific number of rows and columns between the current pixel and its neighbor. This offset is computed in polar coordinates as [ $D \cdot cos(\theta)$ ,  $D \cdot sin(\theta)$ ], where *D* is the length of the vector between the pixels of interest, and  $\theta$  is the orientation of the vector, typically 0, 45, 90 and 135°. In our experiment, the offset was established as the direction perpendicular to the PO (i.e.,  $\theta_{PO-90^\circ}$ ) measured using the structure tensor procedure above described.

The software extracts the correlation value from the GLCM that has been computed with an offset that increases (in every iteration) the D value at the orientation calculated previously. Then, it gets the values of the distances between pixels where the correlation is maximum and computes the mean value. Finally, known the microns subtended by each pixel of the image, the size and the fiber density can be computed.



**Fig. 4.** Example of the calculation of the correlation parameter in an artificial image containing a sinusoidal fringe pattern. (a) Artificial image; (b) intensity profile along the red line; (c) correlation parameter as a function of the distance between pixels.

For a better understanding of this procedure, an illustrative example is presented in Fig. 4. In particular, Fig. 4(a) is an artificial image composed of white (gray value = 1) and black fringes (gray value = 0) with the same nominal width (24 px, as shown by the green arrow inserted). Figure 4(b) shows the profile along the red line drawn in Fig. 4(a), where the distance between two consecutive peaks is 24 px (i.e. the actual width of the bars within the image). Finally, Fig. 4(c) represents the correlation values (computed as explained above) as a function of the distance between pairs of pixels. As expected, the distance between two consecutive peaks is 24 px. This means that, for pairs of pixels separated N\*24 px (with N = 1, 2, 3...), the gray values increase and decrease together. On the other hand, if the distance between pixels is (N\*24) + 12 px, the correlation will be negative, because while the gray value of one of the pixels increases the other decreases accordingly.

#### 3. Results

#### 3.1. Multiphoton images

Figure 5 shows examples of TPEF images of the crystalline lens acquired at the different PMIs used in this work. A simple observation reveals changes occurring with the time after dead. In particular, the structure at 24 h is composed of fairly-aligned (quasi-parallel) fibers. At 48 h, the arrangement is also visible, but compactness turns into a more spaced pattern. After 72 h the tissue begins to disrupt, fibers grow bigger and become much less delineated. Finally, at 96 h fibers are not visible, and a homogeneous structure appears.



**Fig. 5.** TPEF images of the rabbit crystalline lens acquired at PMIs of 24, 48, 72 and 96 h (as indicated).

#### 3.2. Structural organization

As explained above, the organization of the fibers within the tissue has been measured through the structure tensor. The results for two TPEF images acquired at PMIs of 48 and 72 h are presented in Fig. 6. Original images together with the histograms of PO distribution are included for a better understanding. For the TPEF image corresponding to a 48 h PMI, the SD was  $10^{\circ}$ (Fig. 6(b), see inset), which means that the lens fibers are highly organized. Meanwhile, for the image acquired 72 h after death, the value increases up to  $19^{\circ}$  (~2x) which is close to a partially organized structure.

The averaged SD values as a function of PMI for all the lenses involved in the experiment are depicted in Fig. 7. It can be observed that for the 24 and 48 h sets, the quasi-organized spatial distribution of the fibers keeps fairly stable around  $12^{\circ}$  (no significant differences). Despite the loss of fiber delineation, for 74 h there is a slight, but also not significant, increase in SD. However, this value (~17°) is still below 20° which is the limit between organized and partially organized patterns (dotted horizontal line). Moreover, as expected from TPEF images in Fig. 5, the plot shows a noticeable increase in SD (i.e. lower organization) between 72 and 96 h after death, reaching a SD value located closer to a non-organized fibrillar network. This value for a PMI of 96 h is statistically different from those found for 24, 48 and 72 h (p < 0.0001).



**Fig. 6.** TPEF images of the rabbit crystalline lens acquired at PMIs of 48 and 72 h (a, c). The corresponding histograms of PO are also presented (b, d). Values of SD are included for the sense of completeness.



**Fig. 7.** Changes in SD of the rabbit lens as a function of the PMI. Each red symbol corresponds to the mean value across all specimens and locations within a PMI. Error bars indicate the standard deviation. The dotted horizontal line indicates the limit between quasiand partially organized structures. (\*\*\*: p < 0.0001)

#### 3.3. Fiber density

Figure 8 compares the correlation parameter for TPEF images of lenses acquired 24 and 96 h after death. For the former, the plot shows regular cycles, where each peak indicates the maximum correlation between pixels (i.e., the gray values are similar). For the latter, this maximum is remarkable lower and very irregular, which means that the lens structure becomes less regular. Since the resolution of the images is known, the fiber size can be computed. For examples in Fig. 8, the mean size was  $9.1 \pm 0.1 \,\mu$ m and  $13.2 \pm 1.6 \,\mu$ m respectively.

As explained in Methods, once the fiber size is known, the fiber density values can be obtained. Figure 9 shows the density mean values for each PMI. There is a significant decrease in density as PMI increases (R = 0.69, p < 0.001), what represents a progressive denaturation of the crystalline lens structure with the time after death.



**Fig. 8.** TPEF images acquired at two different PMIs: 24 h (a) and 96 h (c). (b, d) Correlation parameter used to measure the fiber size computed from each TPEF image.



**Fig. 9.** Relationship between fiber density and PMI. Each symbol represents the averaged value across all specimens and locations within a certain PMI. Error bars are the corresponding standard deviation. (Best linear fit computed from all individual data: Fiber Density = -0.90\*PMI + 158.23).

#### 3.4. Texture information

Figure 10 depicts the changes in homogeneity and energy as a function of the PMI. For both metrics there are not significant differences up to 72 h after death. However, a statistically significant increase appears for both parameters at 96 h when compared with earlier temporal points. As expected, in terms of homogeneity this behaviour indicates that the crystalline lens structure becomes more uniform. In a similar way, the increase in energy is associated to a more constant distribution of grey level values.



**Fig. 10.** Averaged values of homogeneity and energy computed using the GLCM for the different PMIs. Each bar represents the same as in previous figures. (\*\*: p < 0.001).

120

0.00

0

24

48

PMI (h)

72

96

120

#### 4. Discussion and conclusions

24

48

72

PMI (h)

96

0.50

0

Multiphoton microscopy has provided very useful information when used to image ocular tissues. This technique has been able to detect temporal variations in corneas with swelling and edema [33,34], after chemical burn [35] and in post-crosslinking conditions [36,37]. Moreover, lifetime autofluorescence has also been evaluated in human corneas before transplantation [38]. On the opposite, TPEF imaging analyses of the fibrillar crystalline lens tissue are lacking in the literature.

In the clinical practice, temporal changes in the different ocular structures are important since they might be used as indicators of disease progression, recovery from injuries or surgical outcomes. However, in a particular field such as forensic science, the physical status of some biological tissues at certain time points are often critical since it can be used to estimate the PMI.

Despite it was early reported that ocular tissues can be employed to determine the time of death, cadaveric eyes have been rarely analyzed in-depth for this purpose [39,40]. Postmortem anatomical observations of the retina and the cornea, and biochemical analyses of the vitreous body have been reported to be useful to estimate the time of death, or in some case, to determine the cause of death [39,41–45]. Moreover, physical properties of the lens such as sphericity, histological alterations and absorbance have been also proposed as potential parameters for PMI estimation [22].

It is well-known that establishing the PMI is one of the most complex problems for forensic pathologists. If ocular methods are used, reliable and objective parameters are required to accurately assess the time since death [46]. Ultrasonic pachymetry and optical coherence tomography have been used to explore postmortem changes in the corneal thickness [46–48]. A progressive thickening tendency with a strong non-linear correlation with the PMI was reported. Moreover, the importance of changes in corneal turbidity has also been highlighted [43,49].

On the other hand, to the best of our knowledge, studies of the crystalline lens in postmortem conditions are scarce. Stemberga et al. tested different methods assessing lens opacity after death [21]. Prieto-Bonete and co-authors reported a morphological analysis (macroscopic and histological) of the lens as a function of the PMI (from 24 to 96 h) [22]. Transparency was apparent between 24 and 48 h. This clearly reduced at 72 h and it was minimum at 96 h. Sphericity also decreased with time (differences were found to be statistically significant among all PMIs). Moreover, a qualitative histological analysis showed similar spatial arrangements at 24 and 48 h postmortem. Then, the structure tended to lose its uniformity as the PMI increased. They justified these results on the fact that the location of the lens inside the eye (nourished by the aqueous and vitreous humours) might help to maintain the structural conditions of the lens longer.

Since most of these previous works are mainly based on macroscopic and non-quantitative histological analyses, herein we have gone a step forward through a quantitative characterization of the microscopic structural and texture changes suffered by the lens after death.

TPEF imaging microscopy results reveal that 24 h after death, the tissue presents a fairly well-organized structure. This time point has been used as control reference. The values of SD were around 13° and the PO histogram showed a dominant direction of the fibers. For 48 and 72 h this regular distribution was quite stable. On the contrary, SD was close to 30° for a PMI of 96 h, what indicates that the pattern turns into a partially organized one due to the loss of regularity.

A similar behaviour was found for the two parameters obtained from the GLCM. Both, homogeneity and energy, showed significant differences only for 96 h postmortem. These findings are coherent since at that time point it is clearly visible that the lens tissue tends to present a more uniform structure, where the regular distribution of the lens fibers is close to disappear. These results indicate that these parameters might be useful for PMI assessment within long timeframes.

On the opposite, the density of fibers reveals a linear decreasing dependence with PMI (Fig. 9, slope: -0.90 units/hour). On average, by 96 h, the fiber density will be ~50% smaller than that corresponding to a 24 h. This tendency indicates a progressive disaggregation of the fibers with the time after death. Lens fibers were 8.4  $\mu$ m in size at 24 h. This value is comparable to that reported by Kuszak et al. (7.6  $\mu$ m) [20]. This behaviour confirms the usefulness of this is parameter in forensic PMI estimation.

Our findings partially agree with those reported by Prieto-Bonete and co-workers [22]. Discrepancies mainly occur at PMIs of 72 h where the values of parameters such as SD, energy and homogeneity do not differ significantly from those corresponding to early PMIs. However, we need to have on mind that, unlike previous data, our results are strictly quantitative and have been obtained directly from microscopic images and non-stained specimens. Previous macroscopic and qualitative observations might easily underestimate some changes.

The present experiment, carried out in non-stained fixed samples, represent a first step on using TPEF imaging to explore changes in the lens after death. The visualization of the fibers was also obtained from intact enucleated lenses [17] and more recently in living conditions [18]. This indicates that TPEF microscopy might potentially be used to image the crystalline lens fibers in in-situ conditions. However adaptive optics will be required [18], not only due to the location of the lens inside the eye, but also because the loss of corneal endothelial cells and the progression of the edema after the death [46] will introduce more difficulties when imaging the intact crystalline lens without enucleation.

In conclusion, this study has used TPEF images of the crystalline lens from a rabbit model to objectively monitor structural and texture changes at different PMIs. The lens presented a fairly-well organized pattern up to 72 h, which turns into a homogeneous structure at 96 h, where fibers are hardly visible. No significant differences in SD for PMIs shorter than 72 h were found, although the lens fibers appeared thicker and much less defined as PMI increases. A similar behaviour was found using quantitative texture-related parameters provided by the GLCM. On the opposite, it is interesting to highlight the linear decreasing dependence between fiber density and PMI. These results confirm that TPEF imaging is an accurate and effective method to track the temporal progression of the crystalline lens tissue after death. Moreover, this objective tool might be useful in forensic PMI estimation and complement other existing techniques also based on postmortem ocular measurements.

Funding. Agencia Estatal de Investigación (PID2020-113919RB-I00/AEI/10.13039/501100011033).

**Disclosures.** The authors declare no conflicts of interest and have no proprietary interest in any of the materials mentioned in this article. Portions of this work were presented at the Optica Biophotonics Congress: Biomedical Optics in 2024, paper number JS4A.9.

**Data availability.** Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

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#### **Research Article**

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