

Label-Free Assessment of Human Vitreous Microstructures by Dark-Field, Fluorescence

Lifetime Imaging, Multiphoton and Second Harmonic Generation Microscopy

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Abstract

Purpose: To characterize the microstructure and molecular organization of the human vitreous extracellular matrix using label-free dark-field, fluorescence lifetime imaging microscopy (FLIM), two-photon-emitted autofluorescence (TPEF), and second harmonic generation (SHG) microscopy.

Methods: Vitreous samples were extracted from the posterior and anterior segments of phakic, human donors aged 75 ± 5 years. Fresh samples were imaged by label-free dark-field microscopy, FLIM as well as TPEF and SHG microscopy.

Results: Vitreous samples were initially imaged using dark-field microscopy to provide a preliminary visualization of scattered microstructural elements. Statistical analysis of FLIM result showed consistent fluorescence lifetime signatures across the examined aged donors, indicating a comparable biochemical and structural situation of aged vitreous extracellular matrix. Using advanced phasor plot analysis and pattern matching algorithms, distinct fluorescence lifetime signatures corresponding to different collagen types, revealing spatial heterogeneity within vitreous collagen fibers were identified. Shorter lifetime components (approximately 3.5 ns) localized to inner fiber regions matched collagen type II-associated SHG signals, while longer lifetime components (approximately 4.5 ns) within outer fiber bodies were consistent with collagen type IX.

Conclusions: This multimodal imaging approach provides novel insights into the molecular architecture of the vitreous body and establishes FLIM combined with phasor and pattern matching analyses as powerful tools for studying ocular extracellular matrix changes in health and disease samples.

Key words: Vitreous body, fluorescence lifetime imaging microscopy, two-photon-emitted autofluorescence microscopy, second harmonic generation microscopy, extracellular matrix

Introduction

The vitreous body, a soft and transparent hydrogel, contributes to various forms of optical, mechanical, and biochemical homeostasis in the eye.¹ Despite its importance, the vitreous has historically been one of the least studied tissues of the eye, primarily because of its high water content (98–99%)² and delicate extracellular matrix (ECM), which make it structurally fragile and optically challenging to investigate. Proteins, cells, and salts constitute the remaining 1% of the vitreous body.^{3,4} Within this fraction, ECM (e.g. glycosaminoglycans (GAGs)) and collagen have been extensively studied for their roles in maintaining the morphology and structural integrity of the vitreous. GAGs are large, complex carbohydrate molecules composed of repeating disaccharide units. The four main GAG groups include hyaluronic acid (HA), heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS). In the human vitreous, collagens are primarily organized into fibrillar structures composed of a mixed collagen population, including types II, IX, V/XI, and VI, with type II collagen accounting for the largest proportion (~60-75%).⁵ These macromolecular constituents of the vitreous together form a finely tuned network that provides mechanical support, viscoelasticity, and transparency.⁶ Conventional imaging modalities such as slit-lamp examination, optical coherence tomography (OCT), dark-field illumination, or ultrasound provide valuable morphological and structural information about the vitreous and its interface with the retina but lack molecular specificity.^{7,8} They are generally unable to distinguish among matrix components (e.g., different collagen types, GAGs, and modified proteins) or to detect early biochemical changes associated with aging or disease. Fluorescence imaging, particularly autofluorescence methods, can reveal the presence of intrinsic fluorophores such as oxidized or crosslinked collagens/ advanced glycation end-

products (AGEs), but intensity-based fluorescence is often confounded by light scattering, photobleaching, and overlapping emission spectra.⁹

Beyond measuring fluorescence intensity, additional insights can be gained from examining fluorescence decay time that is an intrinsic characteristic of a fluorescent marker sensitive to changes in the microenvironment, such as physiological conditions in living cells.^{10–12} Unlike fluorescence intensity, decay time is largely unaffected by fluorophore concentration, excitation wavelength, and exposure time. Environmental sensitivity and independence from fluorophore concentration make fluorescence decay time a complementary method to fluorescence intensity measurements. Fluorescence Lifetime Imaging Microscopy (FLIM) leverages fluorescence decay time as a parameter, directly correlating it with physiological changes.^{11,13}

Although FLIM is highly powerful, extracting and interpreting lifetime information from FLIM data is analytically challenging. This complexity arises due to the multi-dimensional nature of the raw data and the computationally demanding algorithms required for FLIM analysis.¹⁴ Phasor plot analysis as a fit-free technique, on the other hand, has been used extensively to produce quantitative information of multiplexed imaging.¹⁵ Instead of fitting fluorescence decay curves to exponential functions, the phasor approach transforms the lifetime data into a frequency-domain representation, allowing for intuitive visualization and differentiation of molecular environments.^{16,17}

Second harmonic generation (SHG) is a nonlinear optical process in which two photons interact with a non-centrosymmetric structure to produce a new photon at exactly twice the excitation frequency. Unlike fluorescence, SHG does not involve electronic excitation or photon absorption, making it an inherently label-free and photostable signal source.¹⁸

In biological tissues, SHG arises predominantly from highly ordered structural proteins such as collagen and myosin, providing structural contrast that complements the lifetime-based biochemical information obtained from FLIM.

In this work, we applied FLIM, two photon–emitted autofluorescence (TPEF), and SHG microscopy to investigate the vitreous body in a fully label-free manner. To our knowledge, this is the first study that combines lifetime-resolved endogenous fluorescence with SHG-based structural contrast to characterize the intrinsic optical signatures of the vitreous. By integrating phasor analysis and pattern-matching unmixing, we demonstrate that multiplexed label-free imaging can reveal distinct biochemical and structural features of the vitreous body that have not been accessible previously.

Figure 1 summarizes our central concept that combining darkfield, multiphoton excitation (MPE) imaging and FLIM enables label-free visualization and differentiation of vitreous collagen fibers.

Materials and Methods

Vitreous Body Dissection

All procedures, including the acquisition and sectioning of the donor eyes, were performed in accordance with the tenets of the Declaration of Helsinki for the Use of Human Tissue and were approved by the local ethics committee (EC number: A 2025-0027). The dissection was carried out as previously reported.¹⁹ In brief, human cadaver eyes donated for research purposes were obtained from The German Society for Tissue Transplantation (DGFG) with written consent from the donors' next of kin. Following an initial incision into the sclera 3.5 mm posterior to the limbus, a circular 360° incision was made using surgical scissors to remove the anterior segment of the eye. The vitreous body was then carefully luxated with blunt forceps. Vitreous samples were obtained from five phakic donors without known systemic or ocular disease (mean age 74.4 ± 3.44

years, range 70–80; male:female = 2:3). The mean postmortem interval was 24.6 ± 12.5 h (range 11–47 h). A vitreous section (~20 μ L) was taken from the posterior and anterior segment of the vitreous body using a scalpel and placed between a microscope slide and cover slip, sealed airtight using nail polish. Intraoperative vitreous samples were limited due to the destruction of the vitreous network structures following fragmentation by the vitrectome and were unusable for further analysis.

The number of donors reflects the limited availability of post-mortem human eyes that met our inclusion criteria (phakic, no known ocular or systemic disease, short post-mortem interval) during the study period. Consequently, the present work was designed as an exploratory proof-of-concept study rather than a statistically powered population analysis.

Dark-Field Microscopy

A customized condenser with ultrahigh resolution (CytoViva® 130, Auburn, AL, USA) connected to a research grade optical microscope (Olympus, BX41, Tokyo, Japan) and a powerful light source (EXFO120 PC, Photonic Solution Inc., Mississauga, ON, Canada) were used for high-resolution optical dark-field microscopy of vitreous sections, visualized with a 60 $\times$ objective. Digital images were captured with a cooled camera (model XLMCT, Dage-MTI, Michigan City, IN, USA) and DageXponent software (version 1.3, Dage-MTI, Michigan City, IN, USA).

Multiphoton and SHG Microscopy

An upright FVMPE-RS multiphoton microscope (Evident, Hamburg, Germany) was used for TPEF and SHG imaging. Dual-line excitation at 1040 nm and 740 nm was achieved using a femtosecond laser system (InSight DS DUAL-OL, Spectra-Physics, USA). Scanning was performed with a Galvano scanner across 1024×1024 pixels, with pixel dwell times ranging from 10 to 40 μ s. The spatial resolution, adjusted by digital zoom, ranged from 0.15 to 0.5 μ m/pixel,

utilizing a 25× water immersion objective (XLPLN25XWMP2, Olympus, NA 1.05). Emission signals from TPEF and SHG were detected in two separate channels (460–500 nm for TPEF and 520–560 nm for SHG) using cooled gallium arsenide phosphide photomultiplier tubes. Image post-processing was carried out with Imaris Viewer (v10.2.0, Oxford Instruments).

FLIM Setup

For time-resolved fluorescence imaging, a MicroTime 200 time-resolved confocal fluorescence microscope (PicoQuant, Berlin, Germany) was used, consisting of an inverted microscope (IX 73, Olympus) equipped with an Olympus UPlanSApo 60× /NA 1.2 and water immersion objective. A ps-pulsed diode laser (LDH-D-C-442, PicoQuant, Berlin, Germany) operating at 442 nm with a pulse width of <90 ps was used as an excitation source. The repetition rate was adjusted at 20 MHz. The laser beam was guided towards the objective using the dichroic mirror zt440rdc (AHF Analysentechnik, Germany). The luminescence was directed through a 50 µm pinhole and detected with a hybrid photomultiplier detector assembly (PMA Hybrid 40, PicoQuant, Berlin, Germany). For the separation of luminescence and rejection of the excitation light, a long-pass filter 450LP was used (AHF Analysentechnik, Germany). Time-resolved luminescence recordings were performed in the time-correlated single-photon counting (TCSPC) mode using a HydraHarp 400 (PicoQuant, Berlin, Germany). In all experiments, the laser power was adjusted to achieve average photon counting rates $\leq 10^5$ photons/s and peak rates close to 10^6 photons/s when recording images. By preserving photon counting significantly below the maximum counting rates allowed by TCSPC electronics, pile-up effects were avoided. Images were acquired by FLIM bee galvo scanner (PicoQuant, Berlin, Germany) for fast scanning. Data acquisition and analysis were performed by the SymPhoTime64 software version 2.7 (PicoQuant, Berlin, Germany).

FLIM Data Analysis

SymPhoTime 64 is a comprehensive software package that integrates both data acquisition and analysis, serving as the standard environment for controlling PicoQuant hardware and collecting time-tagged time-resolved (TTTR) photon data. It performs classical lifetime analysis with advanced fitting routines, including iterative reconvolution with the instrument response function (IRF), allowing extraction of quantitative lifetimes and relative amplitudes. In contrast, NovaFLIM (PicoQuant, Berlin, Germany) is a more recent, dedicated platform for efficient post-processing of FLIM images. It does not control data acquisition but imports TTTR data collected with SymPhoTime. NovaFLIM offers multiple complementary analysis approaches, including phasor plots and pattern matching, alongside exponential fitting, enabling robust and fast processing of complex FLIM datasets. While exponential fitting in NovaFLIM employs mono- or multi-exponential models to fit photon decay curves at each pixel, it remains computationally intensive and sensitive to photon statistics, especially in heterogeneous biological samples such as vitreous. Together, these tools provide powerful and flexible solutions for FLIM data acquisition and in-depth analysis.

For quantitative comparison of collagen lifetime, we analyzed four non-overlapping fields of view per donor. Fields were selected from regions with well-preserved collagen fibers, sufficient signal-to-noise ratio, and minimal preparation artefacts (e.g., air bubbles or sample motion). All fields were acquired using identical imaging settings. For each field, an intensity-weighted mean lifetime of the collagen fiber pattern was obtained by pattern-matching analysis in NovaFLIM, and these values were summarized per donor.

Phasor Analysis

Phasor approach in FLIM uses the reciprocity principle, allowing direct correlation between image pixels and their corresponding phasor points. Selecting a region of interest (ROI) in either the phasor plot or image enables identification of lifetime components, even if spatially separated. This method can detect minor populations often missed by conventional analyses.²⁰ Pattern-matching approaches in FLIM data analysis are conceptually related to the phasor method, as both techniques classify or segment FLIM data based on characteristic lifetime features. In the phasor approach, lifetime mixtures are mapped to specific positions in the phasor plot through linear combinations of decay components. In contrast, pattern matching does not rely on predefined decay patterns. Instead, patterns are defined based on proximity in the phasor plot, where pixels with similar lifetimes are grouped according to the closest mapped phasor locations. Importantly, the user can control the number of patterns to be identified within a FLIM image, allowing flexible and data-driven segmentation.

Results

Dark-field Images

Dark-field microscopy of vitreous samples from 70- and 80-year-old donors indicated in Figure 2, revealed scattered fibrillar and particulate structures associated with age-related vitreous changes. However, the information obtained was limited to surface-level scattering features. Images show the presence of aggregated ECM fibers within the vitreous of 70- and 80-year-old donors. These fibers appeared as bright, scattering structures, consistent with previously reported age-related condensation of vitreous collagen networks, although the limited number of donors in this study does not allow us to draw firm conclusions about age-dependent changes in fiber concentration or organization. While these fibrillar condensations were detectable, their detailed structure and biochemical composition could not be resolved with this technique.

Aggregates occupied ~15-25% of field area (estimated), providing a qualitative indication of pronounced fiber clustering in aged vitreous samples, but lacking molecular contrast provided by subsequent FLIM analysis.

FLIM Images

FLIM images from donors aged 72, 74, and 76 years are presented in Figure 3. Due to differences in the original acquisition zoom, the fields of view differ between panels; therefore, we provide clear scale bars for each image to facilitate qualitative comparison. In all cases, collagen fibers were clearly visualized, appearing either as oriented linear structures or as wavy fibrillar networks, consistent with the known morphology of vitreous collagen.⁵ Notably, no detectable autofluorescence was observed in the surrounding vitreous gel matrix, indicating that the fluorescence signal arises predominantly from the collagen component.

To apply statistical analysis, the average fluorescence lifetime of collagen fibers identified in Figure 4 was extracted using the pattern matching algorithm in NovaFLIM. Figure 4 summarizes the intensity-weighted mean lifetime of collagen fibers for three donors aged 72, 74, and 76 years. For each donor, four non-overlapping FLIM fields were analyzed, yielding image-level lifetime values that were then averaged per donor. The resulting donor-averaged lifetimes were 4.36 ± 0.34 ns, 4.30 ± 0.24 ns, and 4.34 ± 0.14 ns for the 72-, 74-, and 76-year-old donors, respectively (mean $\pm$ SD of four fields). Detailed phasor plot and pattern matching analysis of images are represented in supplementary material (table S1-S3). For completeness, boxplots of all FLIM-derived parameters (a_1 , a_2 , τ_1 , τ_2 , τ_{int}^{P1} , τ_{int}^{P2}) are provided in Supplementary Figure S1, highlighting modest age-related differences compared to the intra-group variability in these metrics.

MPE Images

The MPE images reveal extensive fibrous networks with varying degrees of organization and density across the examined donors (Fig. 5). Notably, TPEF signal is more abundant than SHG. SHG signal, while sparser, shows characteristic spatial overlap with TPEF fibers, with SHG frequently localizing to the core or central axis of TPEF-marked fibers. Because SHG intensities were relatively low compared to TPEF and the images were acquired with pixel sizes optimized for extended fields of view rather than for precision intensity measurements, these MPE/SHG data were evaluated qualitatively rather than by automated intensity-based segmentation.

The fiber morphology exhibits at least two distinct phenotypes: curls or wavy fibers with diffuse autofluorescence pattern, and straight, elongated fibers. These structural characteristics vary in relative prevalence across the examined donors, but the limited sample size does not allow us to distinguish donor-specific from age-related differences.

Quantitative Comparison of FLIM And MPE Structural Metrics

To assess the robustness of the FLIM-based structural readout, we quantitatively compared collagen fiber thickness and coagulation area derived from FLIM images with the corresponding metrics obtained from MPE images across the examined donors (Figures 6 and 7).

For fiber thickness analysis, 120 fibers were measured in total, with 20 randomly selected fibers per donor and imaging modality. The specific regions of interest and example images used for these analyses are provided in the Supplementary Figures S2 and S3. Boxplots of fiber thickness values show that FLIM-derived measurements closely follow the MPE-based thickness across all age groups, with similar medians, overlapping interquartile ranges, and mean fiber thickness consistently in the range of approximately 0.5–1.0 μm for both modalities. The occurrence of a few thicker fibers in the 74-year donor in the FLIM dataset reflects localized collagen bundle

structures rather than a systematic modality-dependent bias, as the overall distributions remain comparable between FLIM and MPE.

We further quantified collagen fiber coagulation area per image by sampling five regions of interest in each FLIM and MPE image for all donors between 72 and 78 years of age (Figure 7). In total, 35 coagulations were evaluated per age group and modality, with measurement points chosen randomly throughout the vitreous collagen network while avoiding obvious artefacts. Both FLIM and MPE reveal a broad spread of coagulation area values with similar medians and overlapping interquartile ranges within each donor, indicating that the two techniques capture comparable variability in vitreous fiber aggregation. Notably, the 74-year donor exhibits the largest coagulation areas in both modalities, suggesting that inter-donor differences in coagulation morphology are consistently reflected by FLIM and MPE rather than arising from modality-specific bias. Together, these quantitative comparisons support that FLIM provides structural metrics—fiber thickness and coagulation area—that are in good agreement with established MPE readouts, reinforcing the reliability of FLIM-based morphological assessment in aged human vitreous samples.

Differentiation of Vitreal Collagen

Comparison between TPEF-SHG and FLIM images reveals a clear differentiation of collagen fibers enclosed by surrounding layers. As shown in Figure 8A and 8B, in the vitreous body of 72-year-old donor, the overlap between TPEF and SHG channels exhibits a strong autofluorescence signal and a weaker SHG response. In contrast, analysis of the same sample in the FLIM image using two pattern-matching algorithms combined with the phasor plot reveals two distinct fluorescence lifetimes along the fiber profile: a shorter lifetime of approximately $\tau_1 = 3.5$ ns in

the inner fiber region and a longer lifetime of $\tau_2 = 4.5$ ns in the outer fiber body, see Figure 8C and 8D.

As a complementary illustration of the multimodal workflow, a correlative FLIM–TPEF–SHG measurement was performed on an independent 75-year-old donor who was not part of the main quantitative cohort (Figure 9). In this example, FLIM images color-coded by fast lifetime (1–5 ns) and overlaid with intensity are shown alongside corresponding TPEF and SHG images acquired from the same vitreous regions, highlighting vitreous strands and aggregates with matching fibrillar microstructure and lifetime heterogeneity across modalities. Minor lateral shifts between FLIM and TPEF/SHG panels are observed, with collagen strands appearing slightly displaced in the multiphoton image. This mismatch is attributed to small sample movements and re-positioning during sequential acquisitions in the unfixed vitreous specimen rather than to true structural differences. The measurement is therefore presented as a proof-of-principle demonstration of spatially co-registered FLIM and multiphoton imaging in human vitreous rather than being included in the quantitative analysis.

Discussion

The first-time application of FLIM, TPEF, and SHG microscopy to human vitreous bodies enabled a label-free assessment of the vitreous body's microstructures. FLIM imaging reveals vitreous collagen fibers as either oriented linear structures or wavy fibrillar networks. Since the vitreous matrix is primarily composed of type II collagen (60–75%) with a smaller but significant contribution from type IX collagen ($\approx 25\%$), the observed fibers are most likely attributable to these collagen types.⁵ The aromatic amino acids in collagen within its polypeptide chains, as well as covalent intermolecular crosslinks, contribute to a characteristic autofluorescence signal. This emission is well documented in ocular tissues and connective matrices, and it is sensitive to

biochemical modifications such as glycation, oxidation, and aging. With age, collagen undergoes non-enzymatic glycation leading to the formation of AGEs, which themselves are highly fluorescent. Consequently, the vitreous autofluorescence signal intensifies in elderly individuals and in patients with metabolic diseases such as diabetes, reflecting accumulation of AGE-modified collagen and other extracellular matrix proteins. By contrast, native HA lacks aromatic chromophores and is essentially non-fluorescent. Nevertheless, oxidative stress and glycation in pathological vitreous lead to fragmentation and chemical modification of HA and the surrounding collagen–HA network, which may favor the accumulation of oxidatively modified, fluorescent macromolecules and thus indirectly influence vitreous autofluorescence.^{21,22}

With the statistical analysis applied on the average fluorescence lifetime of collagen fibers identified in vitreous body, a consistent lifetime signature of collagen fibers within the vitreous body was revealed, indicating a comparable biochemical and structural situation of vitreous collagen in old age.

In addition to qualitative visualization, structural metrics derived from FLIM were found to be in good agreement with those obtained from multiphoton excitation imaging, as demonstrated by the fiber thickness and coagulation area analyses. Across donors, FLIM-based thickness and coagulation values closely followed the corresponding MPE measurements, with similar central tendencies and overlapping distributions, indicating that the lifetime-resolved fluorescence signal provides a reliable proxy for vitreous microstructure. This concordance supports the use of FLIM not only for biochemical contrast but also for quantitative assessment of collagen architecture, thereby strengthening the robustness of the proposed multimodal, label-free imaging approach.

Beyond the main donor cohort, a correlative FLIM–TPEF–SHG measurement from an independent 75-year-old donor is included to illustrate that the combined setup can provide

spatially co-registered lifetime and multiphoton data from the same vitreous regions. Despite minor positional mismatches caused by sample motion in the unfixed specimen, these additional images reproduce the characteristic combination of collagen microstructure and lifetime heterogeneity observed in the main dataset and thus serve as illustrative proof-of-principle data for the multimodal workflow rather than as part of the quantitative evaluation.

Building on this uniform lifetime profile, combined FLIM/TPEF-SHG analysis provides deeper insights into collagen subtype distribution and fiber organization. Since collagen types I and II generate the strongest SHG signals, whereas type III produces weaker responses, the short-lifetime component (τ_1) that overlaps with SHG detection is likely associated with collagen type II, in line with the known absence of type I collagen in the vitreous.^{23,24} The longer-lifetime component (τ_2), observed in the outer of the fibers, likely represents type IX collagen, the dominant structural species.^{25,26} The colocalization of SHG signals with TPEF fiber cores indicate a structured arrangement where collagenous cores provide an organized backbone, surrounded by or integrated with autofluorescent protein components. Importantly, FLIM analysis reveals that fibers exhibiting SHG signal display shorter fluorescence lifetimes compared to surrounding autofluorescent structures, suggesting that the collagenous core comprises a distinct biochemical environment with characteristic lifetime signatures.^{23,27}

Fundamental fiber architectures (orientated vs. wavy) remain consistent across the tested age group, though fiber aggregation and clotting increase with age^{2,28,29}. The morphological distinction between wavy and orientated fibers is likely to reflect differences in underlying molecular organization and mechanical properties. Distinguishing the compositional and structural basis of age-related fiber aggregation warrants further investigation and may provide insights into the molecular mechanisms underlying vitreous body aging and age-related changes in tissue

health. However, our donor cohort spans a narrow elderly age range (70–80 years), and each nominal age category is represented by a single individual. The present data therefore characterize structural heterogeneity within aged vitreous samples rather than providing a continuous description of fiber network properties across different ages. As a result, the quantitative results should be interpreted as exploratory, donor-level observations and not as statistically powered age-group comparisons.

While retinal fluorescence lifetimes can already be measured clinically using fluorescence lifetime imaging ophthalmoscopy (FLIO), no comparable in vivo techniques are currently available for the analysis of the vitreous body. In the retina, multiple strongly autofluorescent structures such as photoreceptors, retinal pigment epithelium with lipofuscin granules, and a dense network of blood vessels provide intense and spatially well-defined signals that facilitate lifetime imaging. In our study, we demonstrate that vitreous collagen fibers also exhibit robust autofluorescence with characteristic lifetime signatures ex vivo. However, in the intact eye, any vitreous signal would have to be detected through the overlying crystalline lens and highly fluorescent retinal layers, which poses substantial technical challenges for selective in vivo measurement. Consequently, a key limitation of this study is that measurements had to be performed on postmortem human vitreous samples. Attempts to analyze vitreous specimens obtained intraoperatively during vitrectomy revealed that the native network architecture of the vitreous was disrupted by the mechanical fragmentation introduced by the vitrectome, thereby precluding meaningful structural analysis. Therefore, the present study should be considered a prerequisite ex-vivo characterization of vitreous collagen architecture rather than a directly translatable clinical protocol. By defining lifetime and SHG signatures of vitreous collagen subtypes under controlled conditions, our results

provide essential reference data for the future development of depth-resolved FLIO or multiphoton ophthalmoscopy approaches capable of selectively probing the vitreous compartment in vivo. Future work should therefore focus on translating and further refining FLIM-based multiphoton imaging strategies toward minimally invasive or noninvasive in vivo applications, which could enable longitudinal assessment of vitreous extracellular matrix remodeling under physiological and pathological conditions. Another limitation of our study is the lack of direct immunohistochemical confirmation of collagen subtypes, which will be addressed in future work combining label-free and labeled imaging. We also acknowledge that, beyond the FLIM lifetime quantification summarized in Figure 4 and Supplementary Tables S1–S3, the present study relies primarily on qualitative assessment of multiphoton images. SHG signals in our dataset are comparatively weak and the pixel size and signal-to-noise characteristics were optimized for structural visualization over large fields of view. Under these conditions, automated segmentation and SHG/TPEF ratio calculations would be strongly influenced by noise and could yield misleading numerical values. For this reason, we restricted quantitative analysis to FLIM data and used the MPE/SHG images mainly as structural context. More comprehensive morphometric quantification of TPEF/SHG images, based on acquisition protocols specifically optimized for quantitative analysis and a larger donor cohort, is planned for future studies.

Conclusion

The present study demonstrates that FLIM and TPEF-SHG imaging enable label-free differentiation of vitreous collagen types. Specifically, SHG-positive type II collagen exhibits short $\tau_1 \approx 3.5$ ns lifetimes, while longer-lifetime $\tau_2 \approx 4.5$ ns type IX dominates fiber surrounding, reflecting distinct biochemical environments and molecular organization. Consistent lifetime

signatures (4.30-4.36 ns across 72-76 year old donors) indicate a broadly uniform biochemical environment of vitreous collagen in the examined aged donors, despite pronounced fiber clustering and aggregation. By linking optical lifetime and non-linear signals to collagen architecture, this work establishes a foundation for quantifying age- and disease-related matrix remodeling, offering potential imaging biomarkers for vitreous degeneration and associated pathologies. Future studies should elucidate phenotype-specific aggregation mechanisms to uncover molecular drivers of vitreous aging.

Code and Data

All data in support of the findings of this paper are available within the article or as supplementary material.

Disclosures

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Appendix

Figures and Figure Legends

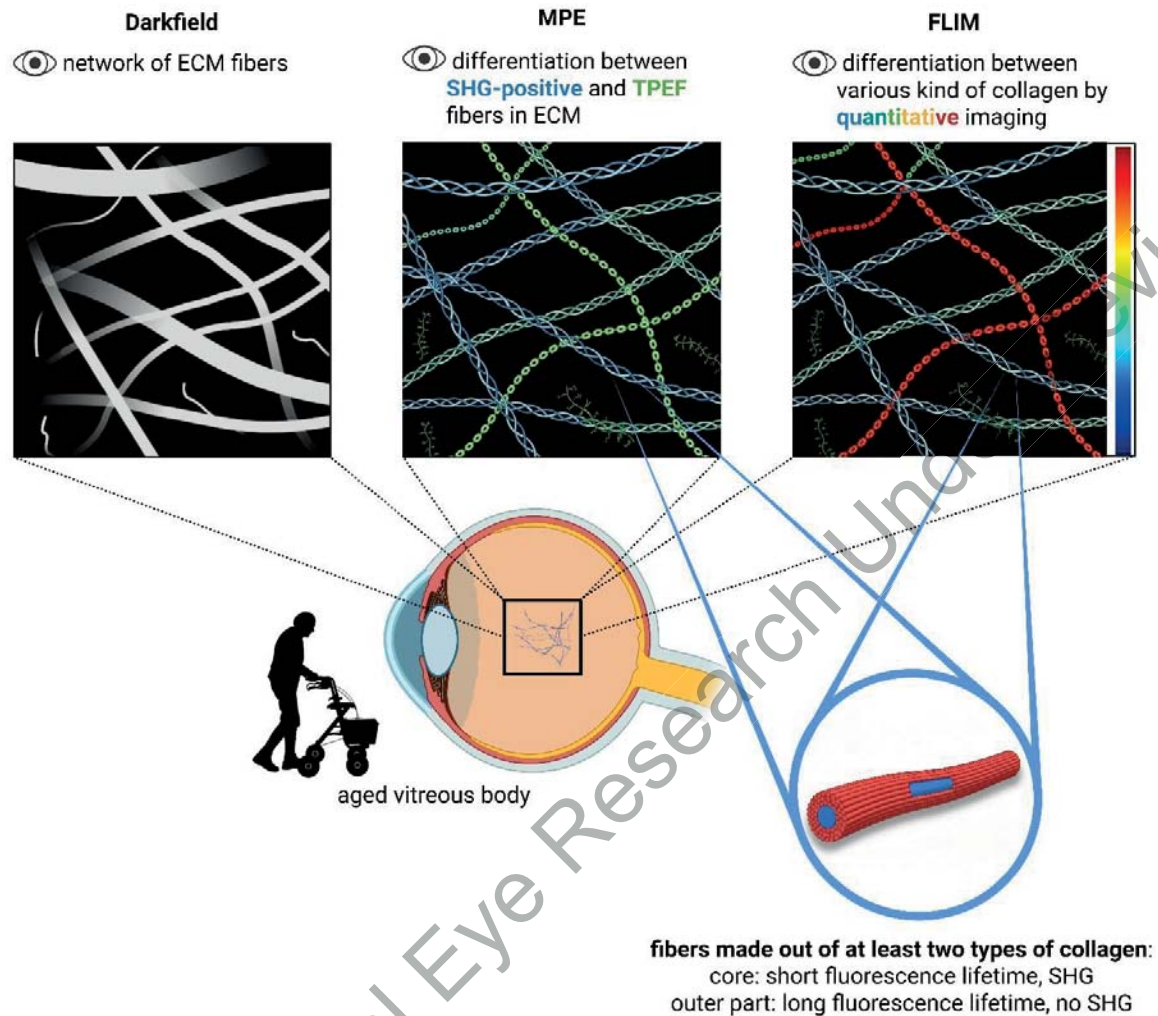


Figure 1. Integrated multimodal label-free imaging reveals collagen architecture in aged human vitreous. Dark-field microscopy visualizes the vitreous ECM fiber network. MPE differentiates SHG-positive (blue) and TPEF (green) signals to show collagen organization. FLIM with quantitative lifetime analysis identifies two collagen types: the fiber core exhibits short fluorescence lifetime and SHG signal (collagen type II, blue), while the outer region shows longer lifetime without SHG signal (collagen type IX, red). Created with BioRender.com.

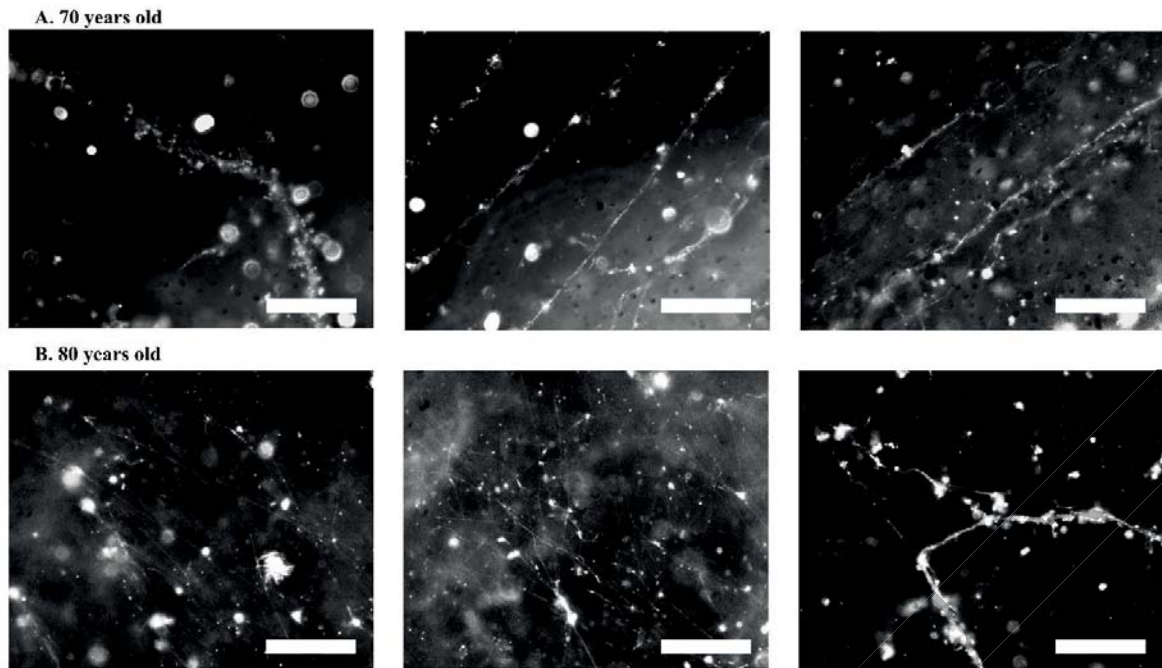
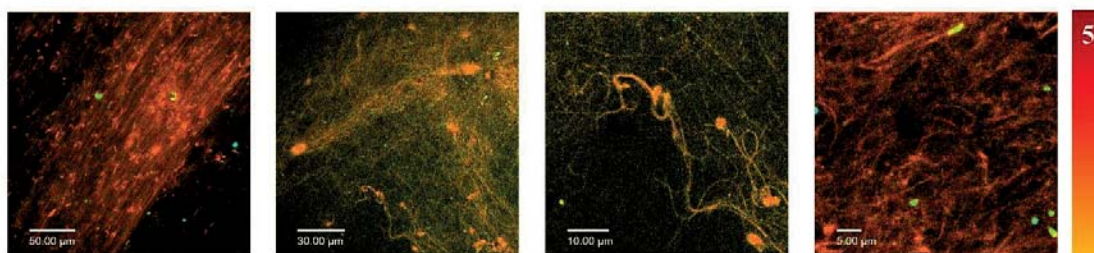
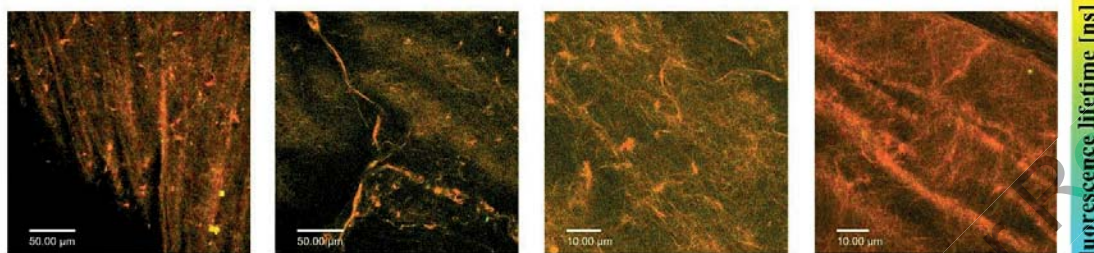


Figure 2. Dark-field images of human post-mortem vitreous body samples of (A) 70 years old and (B) 80 years old donors, scalebar is 50 μm .

A. 72 years old



B. 74 years old



C. 76 years old

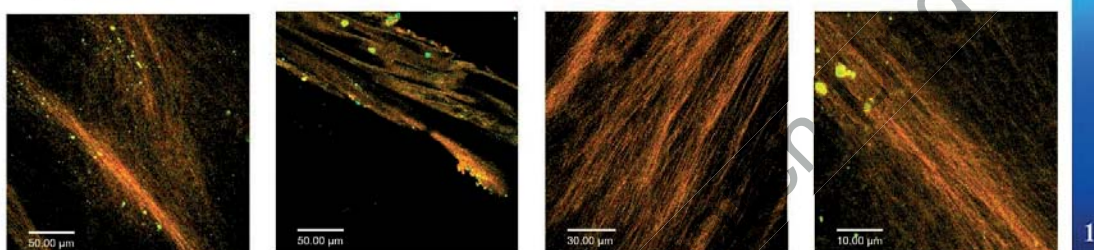
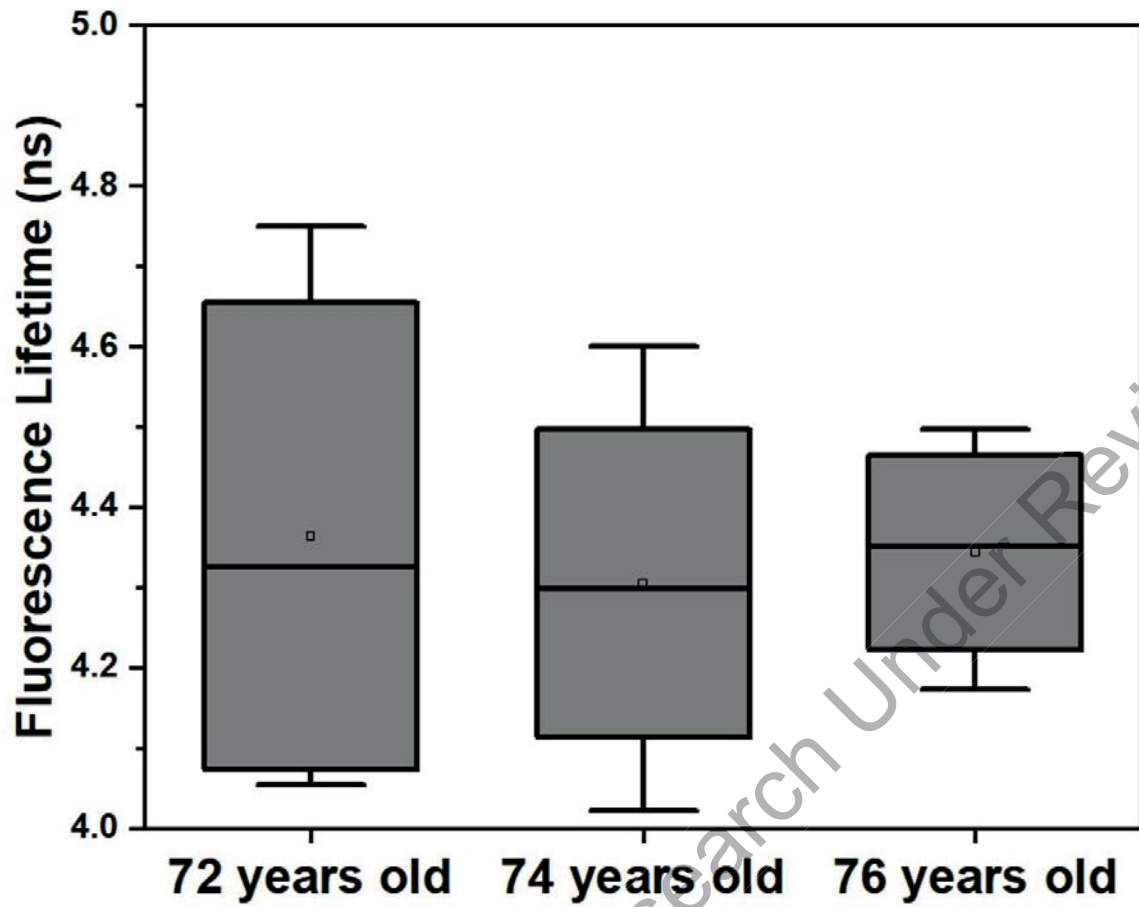


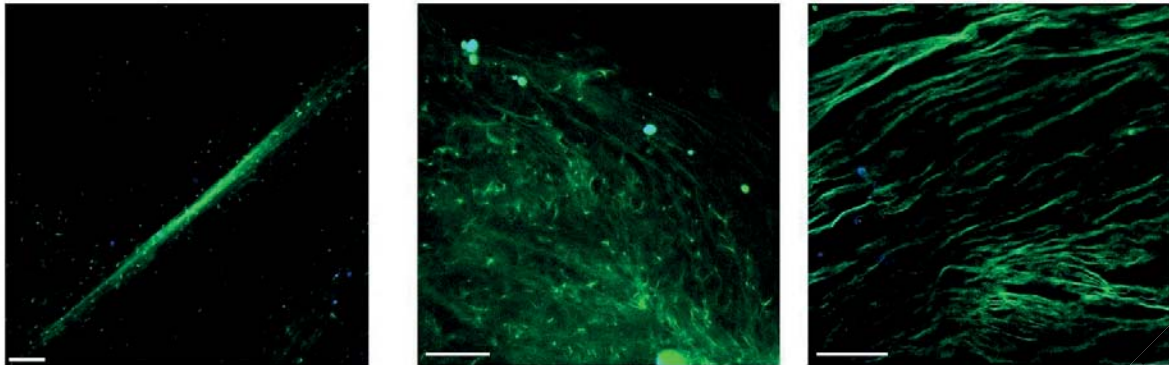
Figure 3. FLIM images showing collagen fiber organization in human post-mortem vitreous body samples from donors aged (A) 72 years, (B) 74 years, and (C) 76 years. The color scale represents fluorescence lifetime variations, with shorter lifetimes displayed in blue and longer lifetimes in red. Scale bar as indicated. Images were acquired with identical optical settings, and field size differences reflect the original acquisition zoom.



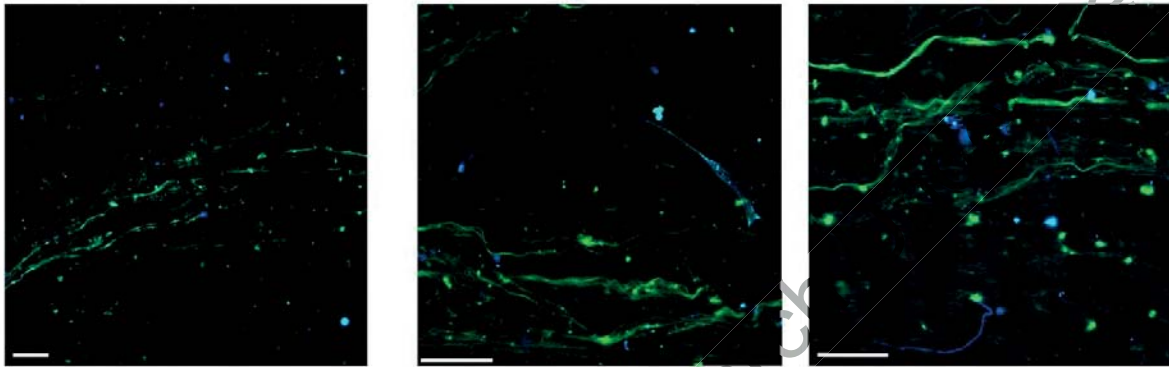
499

500 **Figure 4.** Average fluorescence lifetime of human post-mortem vitreous samples for three donors
501 aged 72, 74, and 76 years. For each donor, four non-overlapping FLIM fields were analyzed (n =
502 4 fields per donor). Data are expressed as mean $\pm$ SD and median.

A. 72 years old



B. 74 years old



C. 76 years old

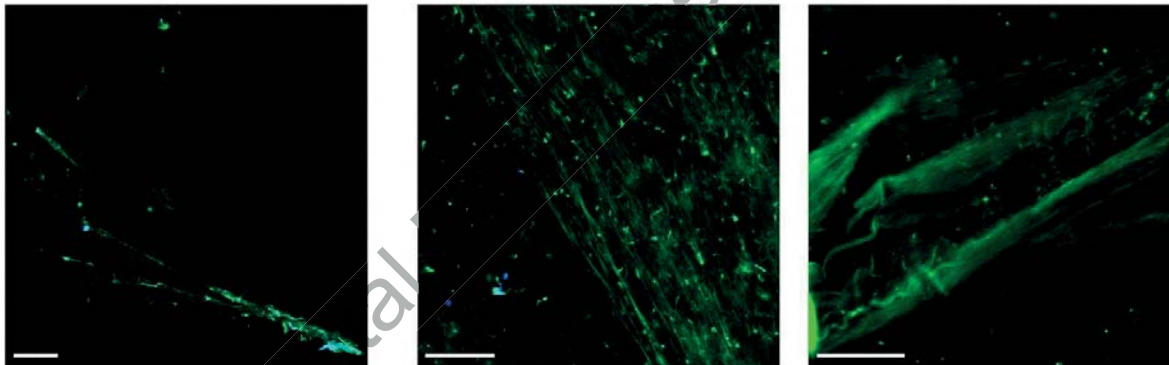


Figure 5. Collagen fiber structure in human post-mortem vitreous samples from donors aged (A) 72 years, (B) 74 years, and (C) 76 years. Autofluorescence is shown on the TPEF channel (green) and SHG signal in blue. Scale bar corresponds to 50 μm .

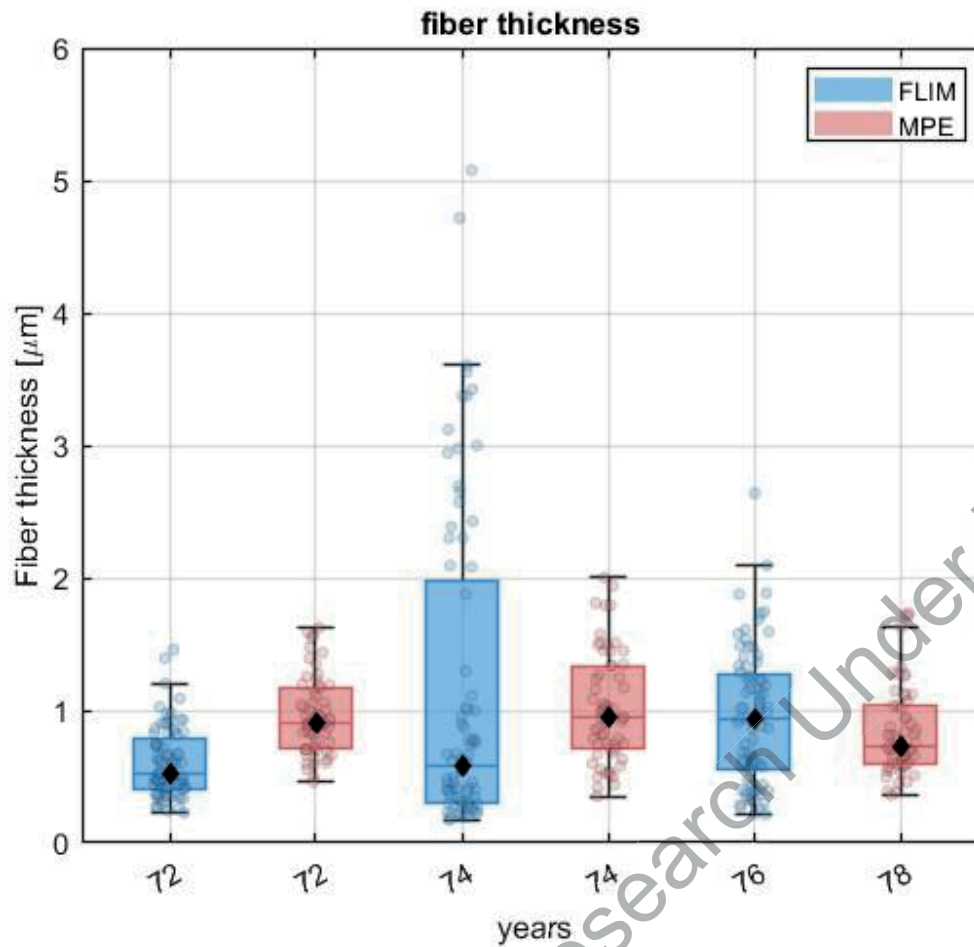


Figure 6. Fiber thickness quantified from FLIM and MPE images across vitreous samples of different donor ages. Boxplots show the distribution of fiber thickness values for each age group (72, 74, 76, and 78 years) measured using FLIM (blue) and MPE (red), with individual data points overlaid and black diamonds indicating the mean.

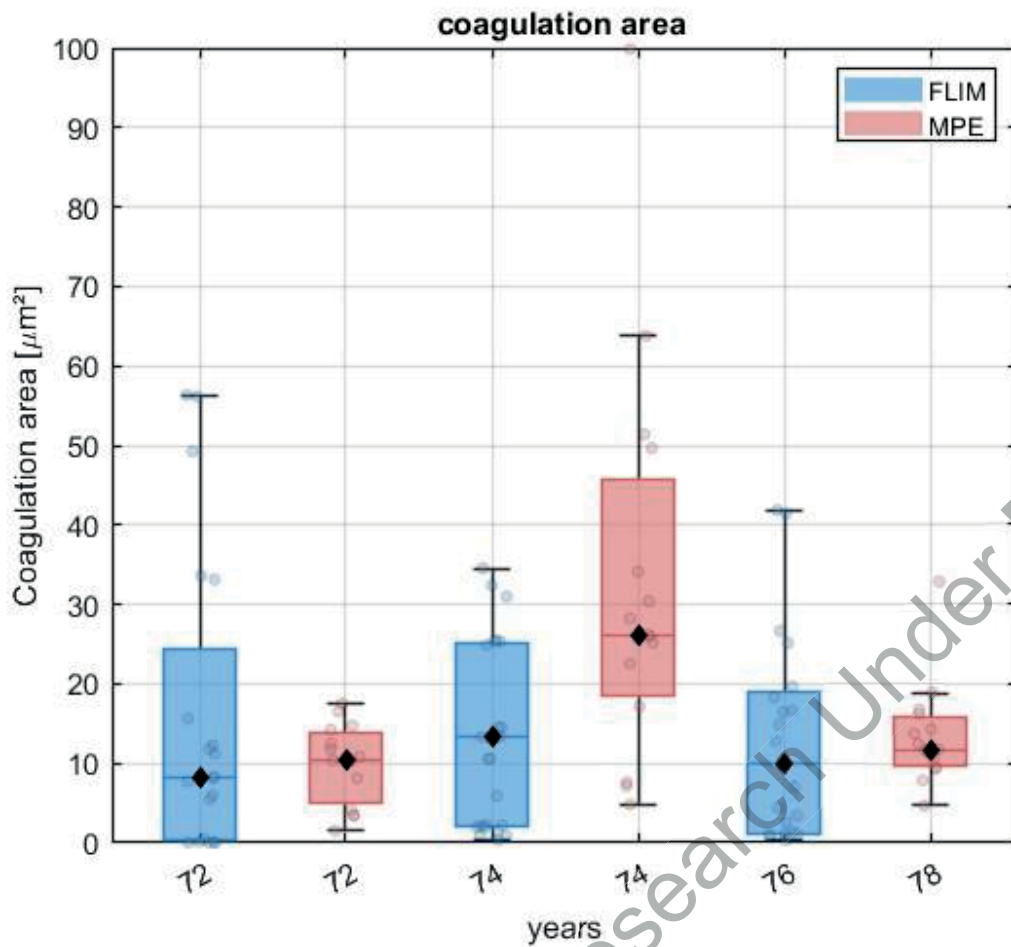


Figure 7. Coagulation area quantified from FLIM and MPE images across vitreous samples from donors aged 72–78 years. Boxplots show the distribution of coagulation area per image (five regions of interest per image) for each age group measured with FLIM (blue) and MPE (red); individual measurements are plotted as semi-transparent circles and black diamonds indicate the mean.

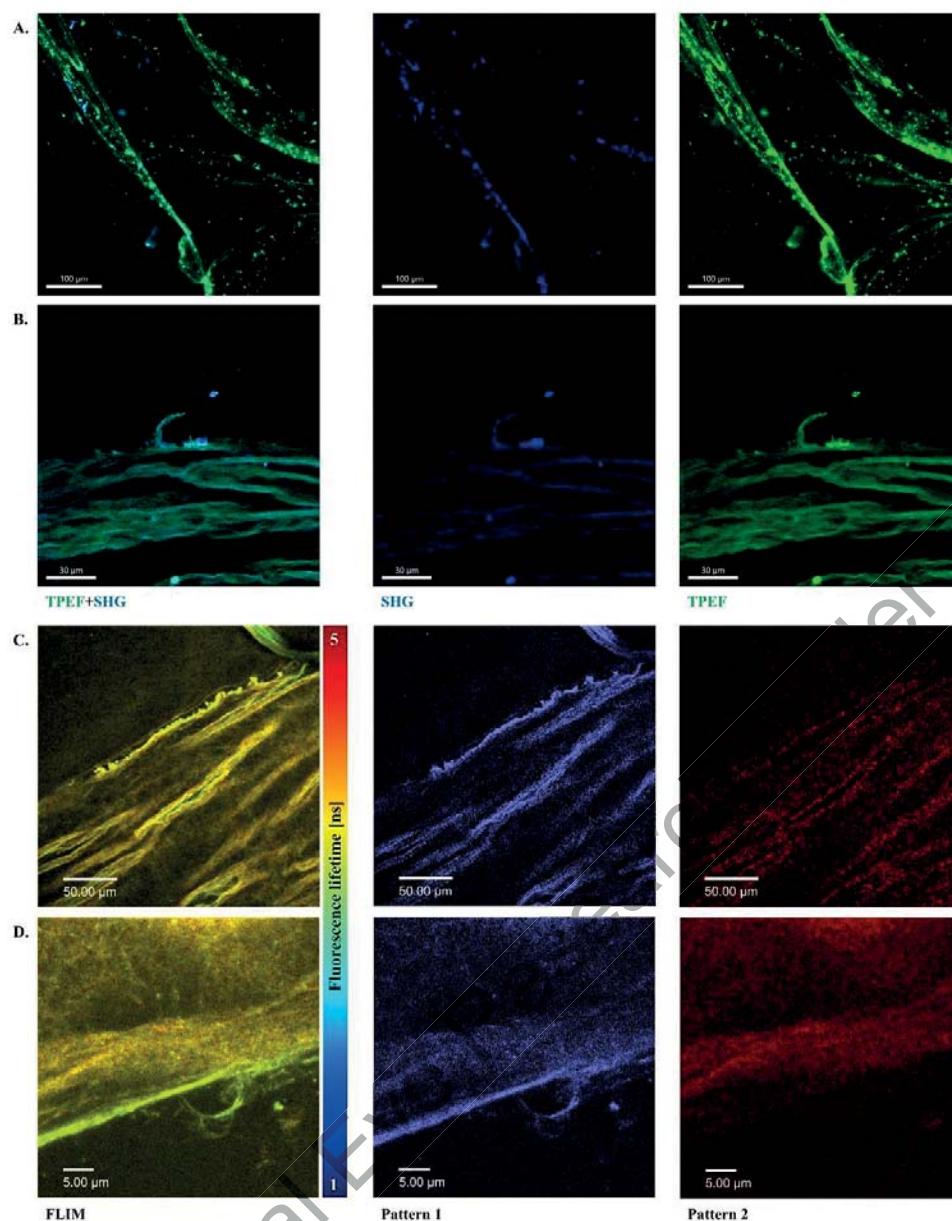


Figure 8. Collagen fiber architecture in the vitreous body of a 72-year-old human donor. (A, B) Left: overlapped TPEF-SHG image; right: single-channel representation showing SHG in blue and TPEF in green. (C, D) Left: FLIM image of the same sample; right: pattern 1 highlighting collagen fibers with distinct fluorescence lifetimes—shorter lifetime ($\tau_1 \approx 3.5$ ns) shown in blue and longer lifetime ($\tau_2 \approx 4.5$ ns) shown in red. Scale bar as indicated.

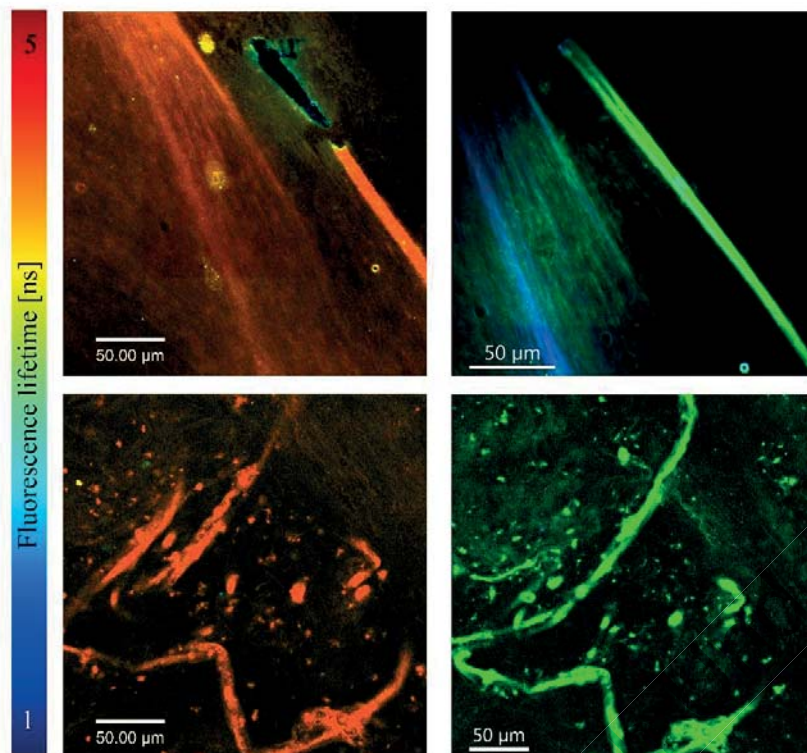


Figure 9. Correlative FLIM, TPEF and SHG imaging of human vitreous from an independent 75-year-old donor. FLIM images color-coded by fast lifetime (1–5 ns) are shown alongside corresponding TPEF (green) and SHG (blue) images from the same regions, illustrating the fibrillar microstructure. Minor lateral shifts between FLIM and TPEF/SHG panels reflect sample motion in the unfixed specimen during sequential acquisitions and lead to small apparent displacements of collagen strands without changing their overall morphology.

Supplementary Material for the following manuscript

**Label-Free Assessment of Human Vitreous Microstructures by Dark-Field,
Fluorescence Lifetime Imaging, Multiphoton and Second Harmonic
Generation Microscopy**

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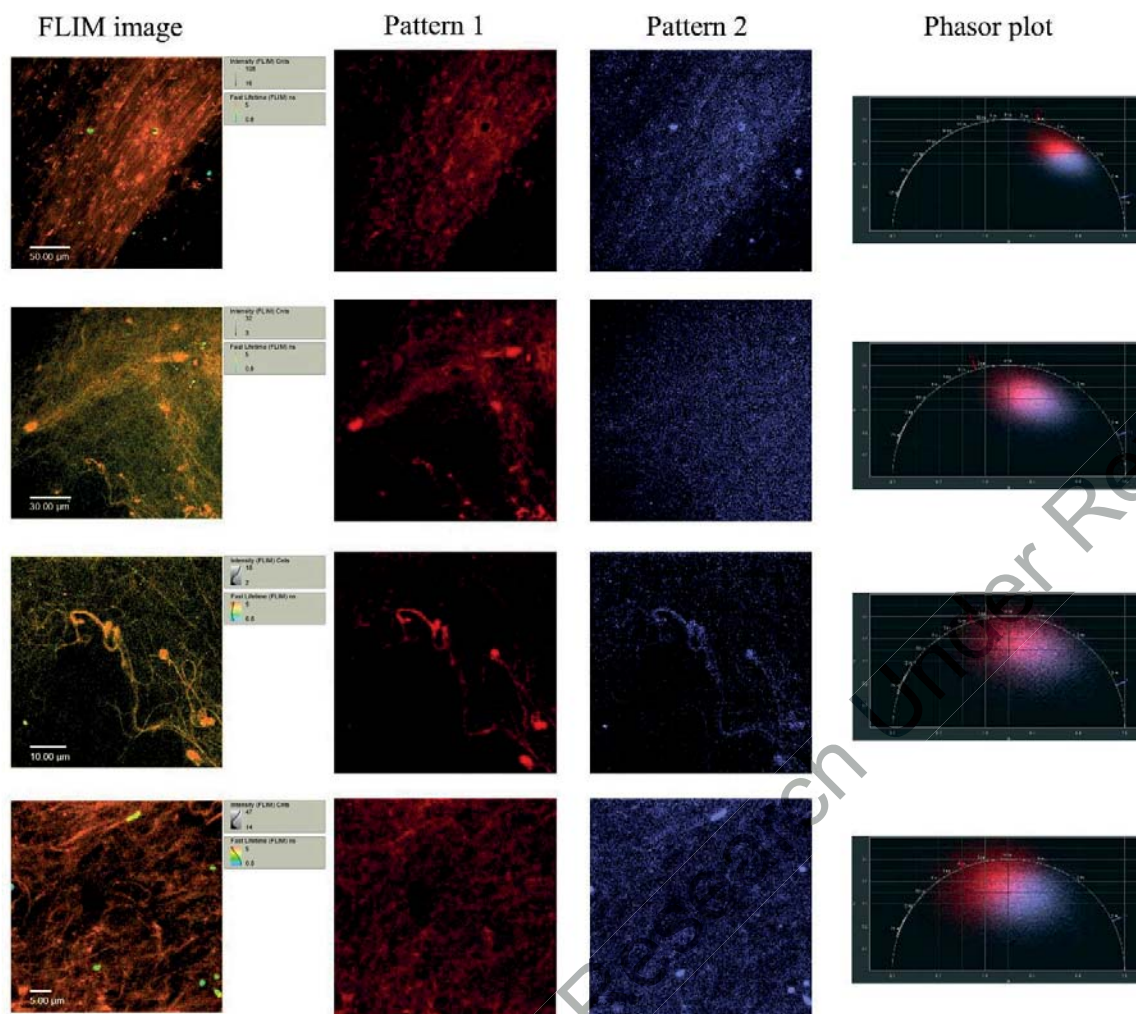
² Department of Life, Light & Matter, University of Rostock, Rostock, Germany

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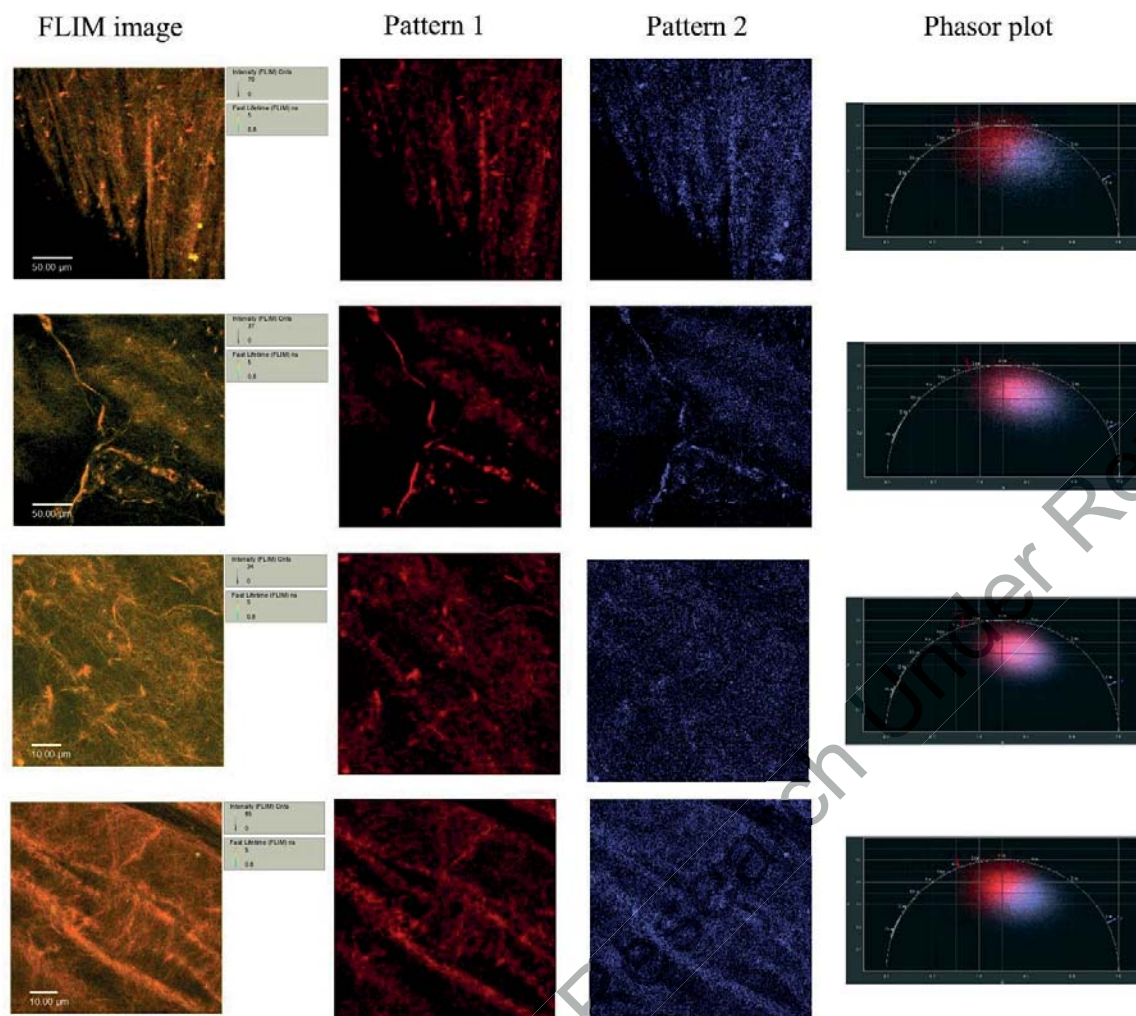
AS and SB contributed equally as last authors.

Corresponding Author:

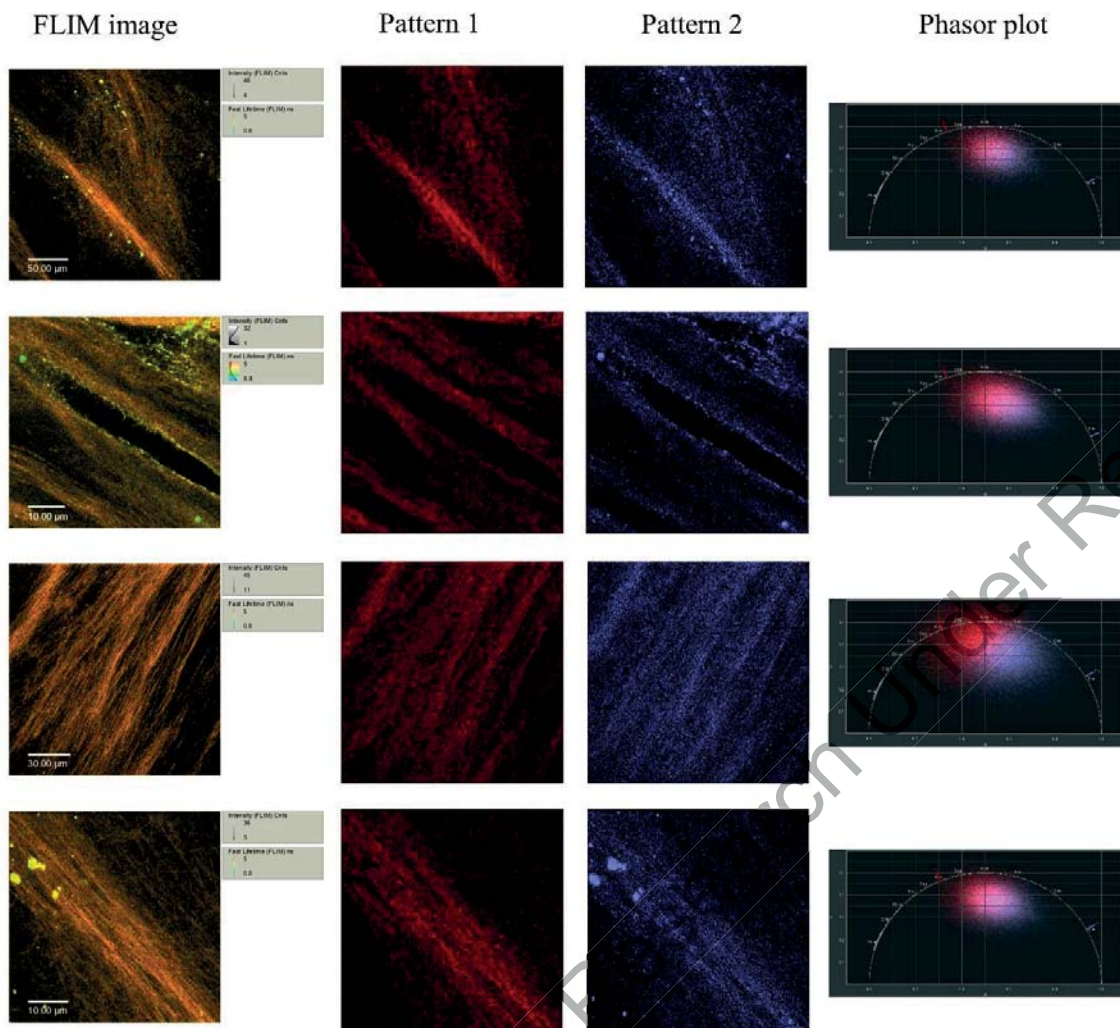
André Schulz, Department of Ophthalmology,
Rostock University Medical Center, Doberaner Straße 140,
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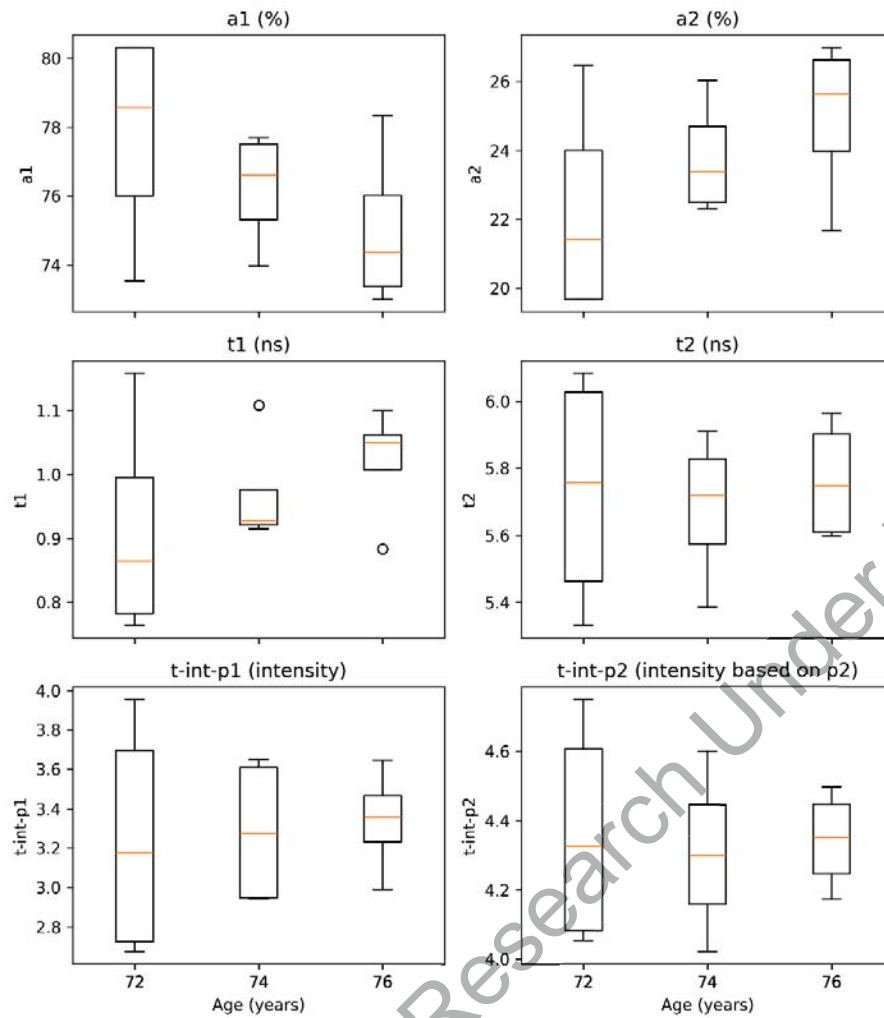
Supplementary Table S1. Pattern matching and phasor plot analysis of vitreous body 72 years old.



Supplementary Table S2. Pattern matching and phasor plot analysis of vitreous body 74 years old.

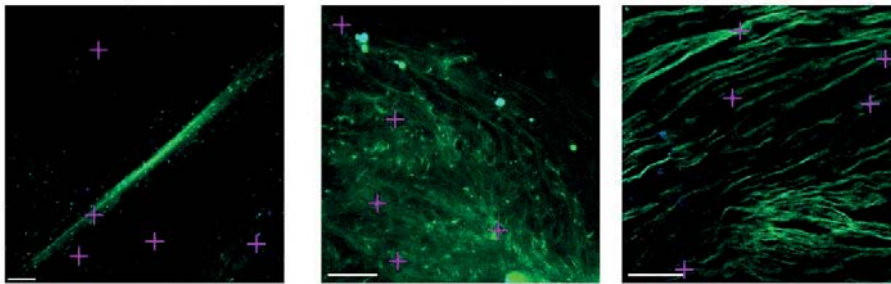


Supplementary Table S3. Pattern matching and phasor plot analysis of vitreous body 76 years old.

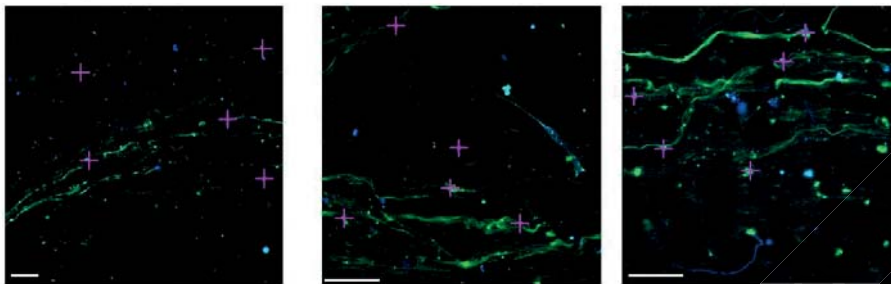


Supplementary Figure S1. Boxplots of six FLIM-derived parameters (a_1 , a_2 , τ_1 , τ_2 , τ_{int}^{P1} , τ_{int}^{P2}) for human vitreous samples from three age groups (72, 74, and 76 years) from FLIM images represented in figure 2. Each box represents four measurements per age group, showing the median (horizontal line), interquartile range (box), and data range (whiskers). The top row displays the fractional amplitudes a_1 and a_2 (%), the middle row shows the short and long lifetime components τ_1 and τ_2 (ns), and the bottom row illustrates the intensity-weighted metrics τ_{int}^{P1} and τ_{int}^{P2} (ns) as a function of age, highlighting only subtle age-related differences compared to the intra-group variability.

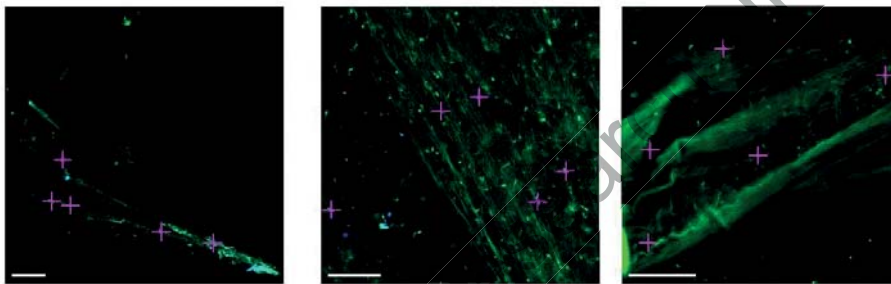
A. 72 years old



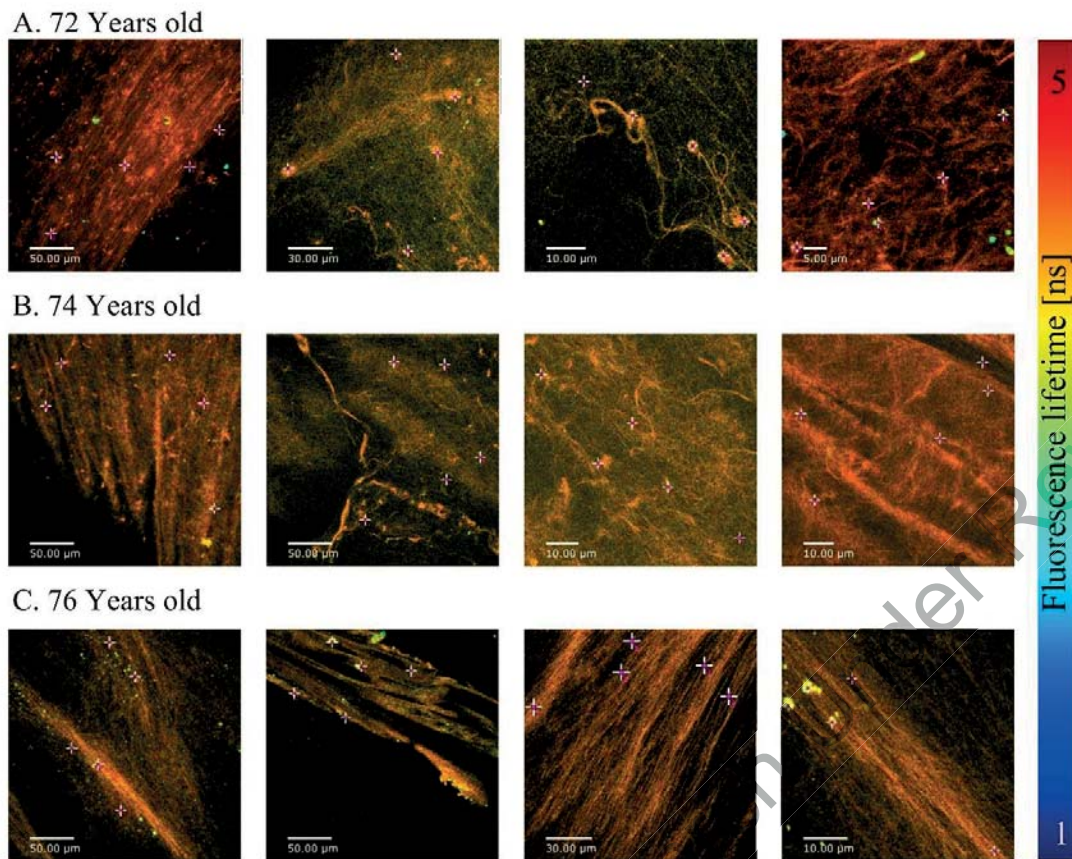
B. 74 years old



C. 76 years old



Supplementary Figure S2. Representative MPE image acquired from the vitreous body sample, with five regions of interest indicated for quantification of collagen fiber coagulation area. Although the exact imaged area differs from the FLIM acquisition, the measurement points were placed in comparable collagen fiber regions, allowing a qualitative and quantitative comparison of coagulation metrics between modalities.



Supplementary Figure S3. Representative FLIM image of the human vitreous body with marked regions of interest used to quantify collagen fiber coagulation area. Five measurement points per image are indicated, illustrating the sampling strategy within the vitreous collagen network while avoiding imaging artefacts and non-vitreous structures.

581 Exponential fitting model

582 The decay equation of emission intensity $I(t)$ at the time (t) describes as:

$$I(t) = \sum_i^n a_i \exp(-t/\tau_i) \quad \text{Equation 1}$$

583

584 Where τ_i is the characteristic lifetime of the decay component and a_i is the subsequent decay
585 amplitude.

586 In order to compare measurements, it is often useful to normalize the decay component amplitude.

587 If a comparison of the concentration of each fluorescing species is required, then normalized
588 amplitude may be useful:

$$a_i = \frac{a_i}{\sum_{i=1}^n a_i} \quad \text{Equation 2}$$

589

590 If a comparison of the contribution to steady-state spectrum (overall fluorescence emission) is
591 needed, then the fractional amplitude can be used, which is the amplitude weighted by the lifetime:

$$f_i = \frac{a_i \tau_i}{\sum a_i \tau_i} \quad \text{Equation 3}$$

592

593 Where f_i is the fractional contribution of the lifetime component.

594 The average lifetime can be defined in two ways. They differ in the way the single decay times are
595 weighed in the averaging:

596 τ_{int} (equation 4) equals the average amount of time for which the emitters (e.g. fluorophores)
597 remain in their excited state after the onset of excitation. τ_{amp} (equation 5) is proportional to the

steady-state intensity. It is applicable when the FRET efficiency is calculated based on the lifetimes of the donor in the presence and absence of the acceptor molecule.

The amplitude average lifetime is characteristic of the fluorophore in the steady state and may be mathematically related to the rate constants. The amplitude average lifetime is commonly used in biological systems where energy transfer between fluorophores occurs, and hence, their lifetime decay is multiexponential due to their heterogeneity and their interaction with their ambient environment.

$$\tau_{int} = \frac{\sum_{i=1}^n a_i \tau_i^2}{\sum_{i=1}^n a_i \tau_i} \quad \text{Equation 4}$$

The intensity average places greater emphasis on the longer lifetimes, reducing the visibility of changes in fractional amplitude and shorter lifetimes. This makes the average appear more stable to changes in the number of components during fitting. The intensity average lifetime is applicable in, e.g., an ensemble of emitters, such as quantum dots embedded in photonic crystals, and semiconductor nanocrystals whose fluorescence is nanocrystal size dependent. Nanocrystals of the same material but of different sizes will emit at different wavelengths and therefore, the average lifetime for the whole excited population of the nanocrystals should be considered ¹⁰:

$$\tau_{amp} = \frac{\sum_{i=1}^n a_i \tau_i}{\sum_{i=1}^n a_i} \quad \text{Equation 5}$$

Phasor plot concept

Phasor analysis simplifies FLIM data analysis by representing signals as complex numbers (phasors) and plotting them in a 2D phasor space, enabling graphical representation instead of complex fitting equations. In the context of FLIM, the phasor method can be applied to both frequency-domain and time-domain measurements, allowing fast and fit-free analysis of fluorescence lifetimes.³⁰

The fluorescence decay information for each pixel can be transformed into phasor space, constructed as a vector or complex number (G and S), derived from the real and imaginary components of the normalized Fourier transform of the intensity decay information $I(t)$ (equations 6 and 7):

$$g = \frac{\int_0^T I(t) \cdot \cos(n\omega t) dt}{\int_0^T I(t) dt} \quad \text{Equation 6}$$

$$s = \frac{\int_0^T I(t) \cdot \sin(n\omega t) dt}{\int_0^T I(t) dt} \quad \text{Equation 7}$$

In which n represents the harmonic number and the modulation frequency ω is now called the laser repetition angular frequency and calculated by multiplying the laser repetition rate with 2π .

The coordinate pair (g, s) defines a unique point for each pixel, which can be plotted in two dimensions to generate the phasor plot—a graphical representation of fluorescence decay data in the complex plane. Alternatively, these coordinates can be interpreted within a vector space, where

633 each phasor is represented as a vector originating from the origin (0,0) and characterized by its
634 magnitude (m) and phase angle (ϕ).

635

$$g = m \cdot \cos \phi \quad \text{Equation 8}$$

636

637

$$s = m \cdot \sin \phi \quad \text{Equation 9}$$

638

Experimental Eye Research Under Review