

AUTOFLUORESCENCE OF TEAR PROTEINS

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Abstract

Tears contain many different proteins which can act as endogenous fluorescence biomarkers. This biological fluid is not used in clinical practice yet. The aim of this work is an experimental analysis of final fluorescence intensity of tear's proteins from right and left eye of healthy subjects by synchronous fluorescence fingerprint. Tears of the healthy subjects exhibited the final protein fluorescence as the fluorescence peak placed at $\lambda = 280 \text{ nm} / \Delta\lambda 70 \text{ nm}$. The fluorescence analysis might be a non-traditional methodology for an early rapid diagnosis of eye diseases in next experimental analysis and in clinical practice.

Key words: tears, tear proteins, autofluorescence

1. Introduction

Tears are interesting biological fluid with a small volume which provides the identification of potential protein biomarkers. Currently, tears are not commonly used clinical and biochemical analytes. The tears contain proteins (mucins, enzymes, glycoproteins, immunoglobulins, etc.), lipids, electrolytes, water and organic solutes. The aqueous layer of the tear film consists mainly of proteins, water and electrolytes while the lipid layer involves several types of lipids e.g. free fatty acids and different esters such as triglycerides, wax esters.

1.1 Proteins

Lysozyme, lactoferrin and tear specific prealbumin (lipocalin) are the major proteins in tears. Lysozyme is the bacteriolytic enzyme that hydrolyzes glycosidic bonds between the saccharide units in the peptidoglycan of bacterial cell walls. Lysozyme occurs besides tears in saliva and breast milk. Its amount increases up to 40 years and then decreases [1].

Lactoferrin has the antimicrobial effect which is the result of binding free iron, thus reducing availability of iron that is necessary for bacterial growth and survival. Another feature includes anti-inflammatory activity

and possibly antioxidant role in protecting the surface of the eye against free radicals. Lactoferrin is an inner barrier to infection.

Tear proteins lactoferrin and lysozyme are co-localized in secretory granules from which they are supposedly secreted together. They have similar features or influence each other activity [2].

Lipocalins have multiple roles in tears: to regulate tear viscosity, to bind released fat, to inactivate virus DNA and to bind iron essential for bacteria. They are family of different proteins with low molecular weight operating outside of the cell and bind to either the small hydrophobic molecules or other macromolecules or specific membrane receptor (LIMR).

Cathelicidins and defensins are the small peptides with the broad-range activity against bacteria, fungi and virus. Moreover, some antimicrobial peptides modulate cell behavior, migration, proliferation and cytokine production. The corneal and conjunctival epithelial cells form and release beta-defensins and cathelicidin (LL-37). *In vitro* studies showed that the peptide LL-37 and human beta-defensin-3 have the most important antimicrobial synergistic activity, which is involved in the modulation of wound healing [3].

Albumin, IgA, IgG, transferrin and secretion components were identified using electrophoresis in combination with immunological methods ELISA and Western blot analysis. By immunological methods have been also identified other proteins: epidermal growth factor (EGF), monocyte chemotactic protein, tissue inhibitor of metalloproteinase (TIMP), angiogenin, chemokines, cytokines, epithelial neutrophil-activating protein and macrophage inflammatory protein [4].

Growth factors, e.g. EGF, are factors that are able to control wound healing of the cornea and conjunctiva, proliferation, differentiation, growth and development of the epithelial cells. EGF is also able to stimulate and modify tear secretion.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases which degrade any type of extracellular proteins. They are also involved in cell proliferation, migration, differentiation, apoptosis and angiogenesis. Against the effect of MMPs act naturally occurring inhibitors of metalloproteinases (TIMPs).

Immunoglobulins are glycoproteins that bind to the antigens (of invasive organisms) with the high specificity and affinity. There are five different classes of immunoglobulins (IgA, IgG, IgM, IgD, and IgE). The immunoglobulins play an important role in the immune system.

Cytokines are proteins or glycoproteins that form the cells in response to specific stimuli. They are multifunctional, short-term, short-range mediators of cellular activity. They are released by T cells and other immune cells. Cytokines have the irreplaceable role in intercellular communication and can induce the growth, differentiation, chemotaxis and/or cytotoxicity.

The chemokines are a small group of cytokines, which influence the chemotaxis of lymphocytes, monocytes and neutrophils that are involved in immune response. Angiotensin II (octapeptide), strong vasoactive component, which is formed by the action of angiotensin converting enzyme, was also isolated from human tears. The presence of these substances presumably affects several physiological functions during angiogenesis in the eye. The exact function has not yet been clarified.

Insulin and insulin-like growth factor (IGF) was also isolated from the tears. It is known that diabetes is associated with dry eye and changed wound healing, what makes insulin important in the physiology of tears.

Lacritin is the protein also found in human tears. It acts as a pro-secretory mitogen (growth factor) in tear secretion and the recovery of the tear surface and epithelial structures.

The proteins are involved in antimicrobial and anti-inflammatory defense of the eye

[5]. They are supposedly important for many other aspects of normal physiology including angiogenesis, biosynthesis, carbohydrate and calcium metabolism, cell adhesion, motility, cell growth, anti-apoptosis and the immune response [1].

1.2 The effects which influence the protein profile of tears

Physiological tear production is 1,2 $\mu\text{L}/\text{min}$, tear film thickness in healthy individuals is 8 – 10 μm . The pH ranges from 7 to 7,3 and the osmolarity is 290 m Osmol/L. The volume of sample that we are able collect from the patient is approximately 5 μL .

The tear is directly exposed to the environment and it adapts to it by changes in the composition, regulation and stability. Hormonal fluctuations as well as diurnal rhythms can change the secretion and/or composition of the tear. The example is the nocturnal rhythm which is responsible for the increased production of tears during the day.

The brain and limbic system also regulate basal tear secretion which is lower in states of fatigue, anxiety, and sleepiness. External environmental factors such as air, air conditioning and labor factors associated with decreased blinking affect the tear stability and consequently may contribute to the relatively high inter-subject variability among normal healthy control volunteers.

Protein composition may vary from day to day, therefore, it is necessary to characterize the variability of proteins [6]. Variation within single days also failed in demonstrating the significant difference. These differences in tear proteome may arise from using different methods of collection of tears which can be realized using either capillary or the Schirmer test papers. The addition of saline to the eye for the collection of tear by "rinsing the eye" is another of the methods that provides the variability of tears protein profiles [7], [8].

1.3 Detection of tear protein

In the past, the structure of the tear film proteins was studied by gel electrophoresis,

Edman degradation and other techniques. They result in the determination of the major tear proteins that are lysozyme, lactoferrin, lipocalin (tear-specific prealbumin), secretory IgA, and lipophilin. In recent years, nearly 500 different proteins were identified in human tears using different methods: mass spectrometry (MS) SELDI-TOF (Surface-Enhanced Laser Desorption Ionization - Time of Flight) MALDI-TOF (Matrix Assisted Laser Desorption Ionization - Time of Flight), high performance liquid chromatography combined with mass spectroscopy HPLC-MS (High Performance Liquid Chromatography - Mass spectroscopy) and other proteomic analysis [9]. The advantages of mass spectrometry over various proteomic methods involve a faster setting and high sensitivity detection of proteins and peptides in the range of nanograms and picograms, respectively. These features find use in the detection of minority tear proteins (concentrations below 0,1 mg/mL) [10].

Native unpurified tear samples include, besides peptides and proteins, other compounds (salts, lipids) which are required to remove by using different chromatographic techniques. Sample editing before profiling of peptide/protein tears can increase their detection and reproducibility.

In addition to mass spectrometry, the two-dimensional gel electrophoresis (2-DE) is currently used. It uses the isoelectric point and molecular weight of the protein to distinguish between the different low molecular weight proteins. Protein staining can be realized by silver, fluorescent colors and chromogenic isotopes. Quantitative analysis uses fluorescent differential display of 2-DE. This technology exploits a combination of several fluorescent analysis for the identification of the different fluorescently labeled proteins using multiple fluorescent labels which are on the same electrophoresis gelside by side. This procedure greatly improves the accuracy, reliability and reproducibility of

measurements. In this technique, each point has its own standard and its position is evaluated by automatic software to identify the protein and its amount. The utility of the method is limited to the ability of separation and operating procedures.

The novel methods with the considerable diagnostic potential are synchronous fluorescence analysis. It determines the total amount of all fluorophores in a sample. The final emitted intensity of fluorescence and the shape of the characteristic fluorescent "fingerprint" is sufficient for comparative diagnostic and rapid, cheap, correct and accurate analysis [11].

2 Material and Methods

2.1. Experimental

Tear collection of health subjects ($n = 10$) was realized by ophthalmologist MUDr. Gabriela Glinská in vacutainer tube in Ophthalmology Clinic, in Košice. Tears were measured immediately by fluorescence synchronous fingerprint. All clinical investigations were conducted according to the declaration of Helsinki principles. Ethical consent for this study has been given by the institutional committee on human research and is compliant with ethical standards on human experimentation and with the Helsinki declaration.

2.2. Materials

Physiological sodium chloride solution (0.9 %) which was purchased from B. Braun, Melsungen, Germany.

2.3. Methods

2.3.1 Collecting of tears

Tear samples of healthy subjects ($n = 10$) from the left eye and right eye were collected by saline flushing method of eyes. The physiological salt solution (100 μ l) was added to the conjunctival sac of eye. The "flushed eye content" was immediately collected by using 100 μ l micropipette in plastic microtubes ($V = 200 \mu$ l).

2.3.2 Synchronous fluorescence fingerprint of tears

The collected samples of tears of the healthy subjects ($n = 10$) were measured

immediately after the collection of tears. The intensity of autofluorescence of the individual control samples was measured in a quartz cuvette (500 μ l volume) by synchronous fluorescence fingerprint (SFF) analysis on Perkin-Elmer Luminiscence Spectrophotometer LS 55, $t = 25^\circ\text{C}$. Excitation spectra were measured in the wave length range $\lambda_{\text{ex}} = 200\text{-}390 \text{ nm}$, $\Delta 10$. The rate of scans was 1200 nm/s, the setting of instrument's excitation slit was 5 nm and emission slit was 5 nm. The measurement of each sample was repeated 10 times. The measured results of fluorescence spectra were processed using the graphic Win Lab software (version 4, 2001). The resulting three-dimensional spectrum of the SFF was created 10 simple synchronous scan spectra of blood serum measured at various $\Delta\lambda$ located in the area with the increment 10 [12]. Measurement of total fluorescence by synchronous fluorescence fingerprint increasingly used in the analysis of a mixture of unknown samples because it provides more information about the mixture in contrast to simple synchronous fluorescence excitation spectra [13].

3. Result and discussion

Tears sensitively reveal changes in the body, thus becomes the object of an appropriate examination by using synchronous fluorescence fingerprint. The fluorophores in tears are the organic compounds mainly proteins which emit the light after the absorption of excitation energy. The synchronous fluorescence spectra are complex of the fluorescence signals of all endogenous fluorophores present in tears. The individual fluorophores can influence each other, but it is not necessary identify signal each signal of individual fluorophores. Synchronous fluorescence spectrum defines the mixture of tear proteins and is considered to be a characteristic "fingerprint" as it is specific for a given tear suspension of the healthy subjects. The most commonly used method of sampling tears is a micro-capillary. By attaching the

microcapillary to temporal edge of the eyelid just to touch the conjunctiva, the very small amount of tears (5 μ l) are collected. This amount of tears is not enough amounts for fluorescence analysis and therefore the collection of tears is realized by rinsing of eye with physiologic solution. A physiological solution (100 μ l) is dripped into the conjunctival sac and then the tears are sampled by micropipette. Sampling the tears by rinsing the eye with the saline solution has benefits in patients with dry eye who have the reduced volume of tears. The

not accurate or correct addition and collection of saline during the collection of the tears can affect the quantitative protein profile (differences in the peak intensities) but not the qualitative composition of the profile (**Figure 1**). The decrease of the fluorescence peak in tears ($F = 452$) from right eye was observed in comparison with tears ($F = 679$) from left eye (at the right) as result of different dilution of tears with physiological solution.

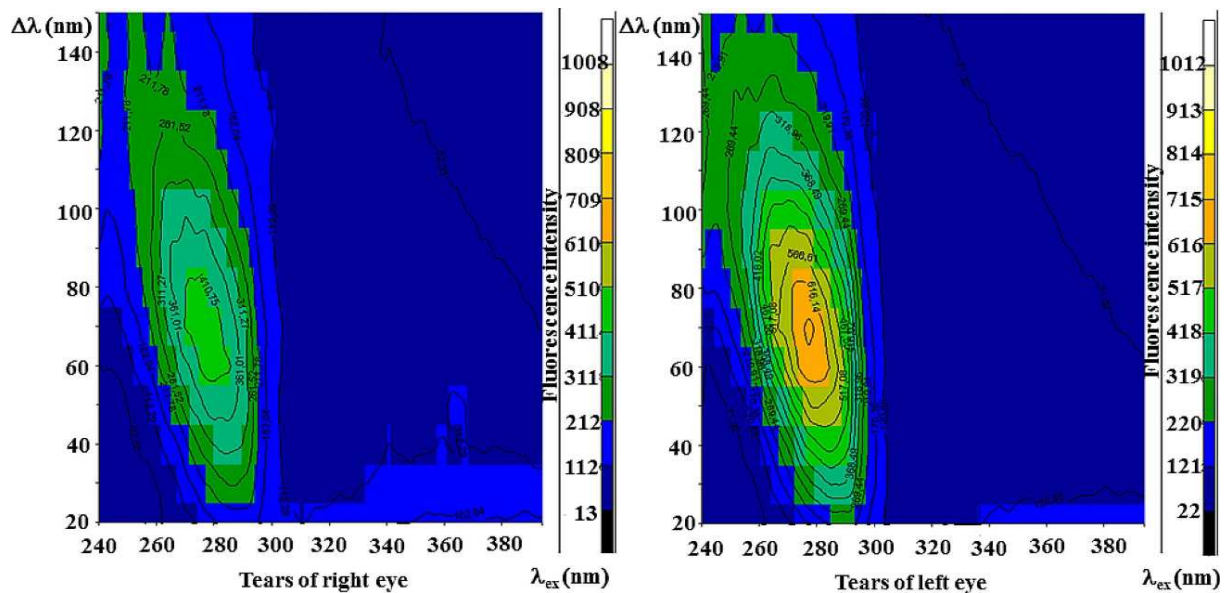


Figure 1: The contour maps of fluorescence synchronous fingerprint (SFF) of tears from right eye (at the left) and of tears from left eye (at the right) of the healthy subject. The fluorescence intensity of fluorescence peak placed at $\lambda = 280$ nm/ $\Delta\lambda = 70$ nm in tears. The decrease of the fluorescent peak in tears ($F = 452$) from right eye was observed in comparison with tears ($F = 679$) from left eye (at the right).

Graphical display of the 3-D maps (**Figure 2**) and the contour maps (**Figure 3**) of synchronous fluorescence fingerprint (SFF) of tears collected correctly from the right and the left eyes of healthy subjects is a profile with the same characteristic shape and revealed similar fluorescence intensity of tears. A detailed analysis of the fluorescence curve is not essential for the analysis (**Figure 4**).

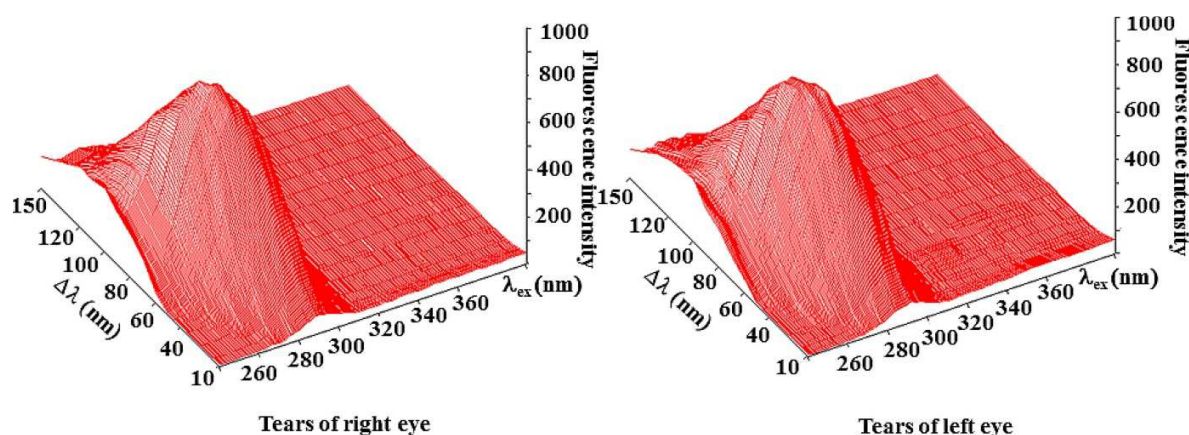


Figure 2: The three dimensional maps of fluorescence synchronous fingerprint (SFF) of tears from right eye (at the left, $F = 756$) and of tears from left eye (at the right, $F = 731$) of the characteristic representative healthy subject. The fluorescence intensity of fluorescence peak placed at $\lambda = 280 \text{ nm}/\Delta\lambda = 70 \text{ nm}$ in tears.

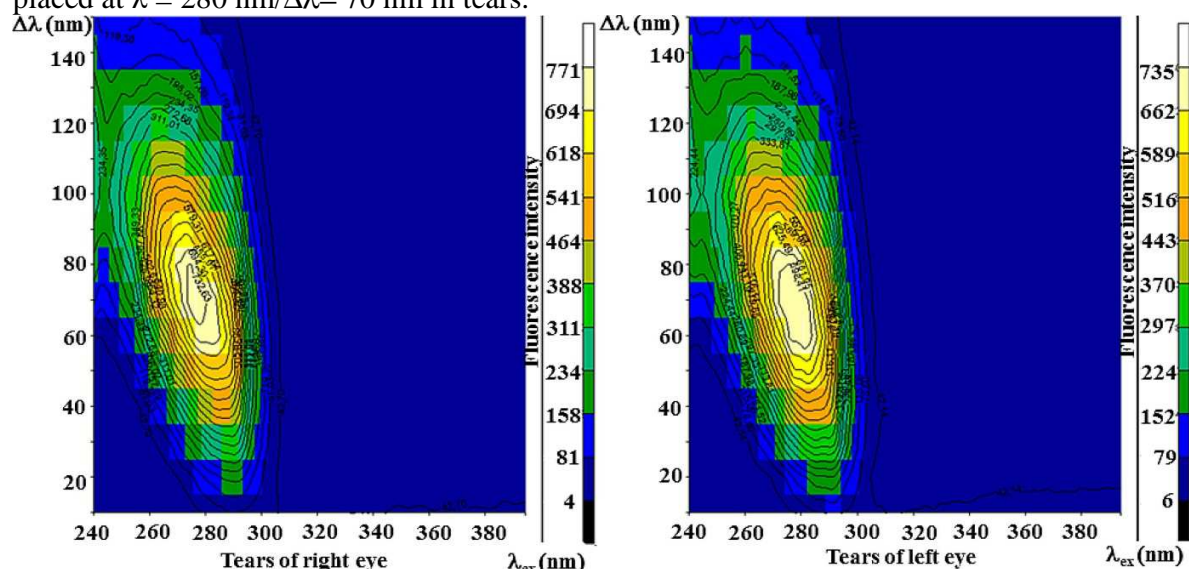


Figure 3: The contour maps of fluorescence synchronous fingerprint (SFF) of tears from right eye (at the left, $F = 756$) and of tears from left eye (at the right, $F = 730$) of the characteristic representative healthy subject. The fluorescence intensity of fluorescence peak placed at $\lambda = 280 \text{ nm}/\Delta\lambda = 70 \text{ nm}$ in tears.

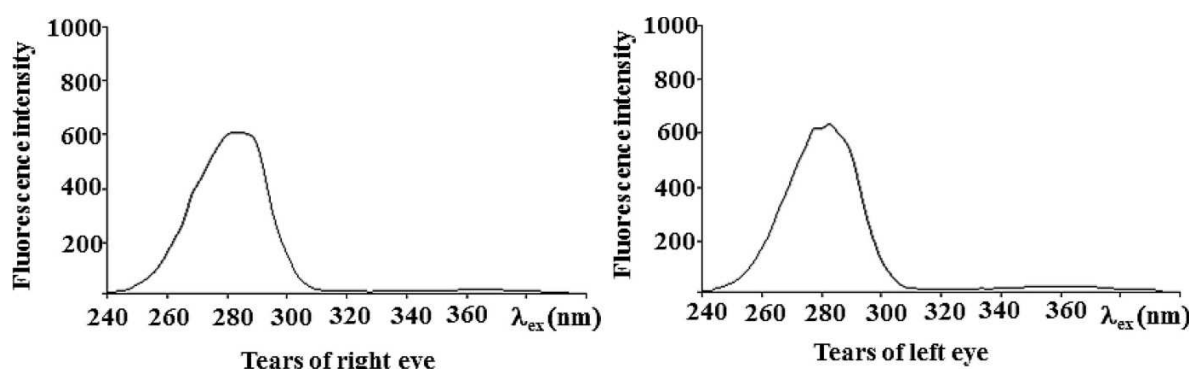


Figure 4: The simple fluorescence synchronous spectra of tears ($\Delta\lambda = 50 \text{ nm}$ is constant difference between emission and excitation spectra) from right eye (at the left, $F = 640$) and of

tears from left eye (at the right, $F = 640$) of the of the characteristic representative healthy subject. The fluorescence intensity of fluorescence peak placed at $\lambda = 280 \text{ nm}/\Delta\lambda = 70 \text{ nm}$ in tears.

The results of this study have showed that there is no significant variability in the protein profile between healthy individuals. Testing of profile bilaterality from the right and left eye of healthy subjects did not show significant difference. Tears of the healthy subjects ($n=10$) shows the fluorescence peak ($\lambda=280 \text{ nm}/\Delta\lambda=70 \text{ nm}$) with the average fluorescence intensity $F = 710 \pm 50$.

A simple comparison of fluorescence profiles of SFF of tears can confirm or disprove the identity of the two mixtures of tears [11], [13 - 16].

Conclusion

This work doesn't describe the biochemical composition of the tears, but rapid analysis and characterization of total intensity of proteins in tears of healthy subjects collected by rinsing of eye with physiologic solution. This saline method allows collecting higher volume of diluted tear sample which is sufficient for fluorescence analysis. Fluorescence analysis is a sensitive and rapid method which is not used in clinical laboratory. The autofluorescence of the tears of healthy subjects was characterized in this study as a rapid analysis of total tear proteins placed at $\lambda = 280 \text{ nm}/\Delta\lambda = 70 \text{ nm}$. The synchronous fluorescence fingerprint of healthy subjects which was investigated in this study will be compared with synchronous fluorescence fingerprint of tears of patients with different ophthalmological diseases in our next experiments. Fluorescence spectroscopy has not been used yet for research of tears but could be a new possibility in early diagnosis of eye pathologies and other diseases.

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