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Microdesiccates produced from normal human tears display four distinctive morphological components

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ABSTRACT

Desiccation of human tears on glass surfaces results in fern-like crystalloids. This phenomenon has been associated with tear normality (Tear Ferning Test, TFT) and is used as a diagnostic aid to evaluate patients with Dry-Eye disease. However, TFT is focused on the assessment of only a minor fraction of desiccated tear samples and considers only the relative abundance and density of fern-like crystalloids. The aim of this study was to characterize morphologically entire desiccated micro volumes of tears from healthy donors. Tear samples were collected from 23 healthy young adult volunteers. Tear aliquots (1-3 μL) were allowed to dry on glass surfaces under ambient conditions of temperature (15-25°C) and relative humidity (40-45%). Dry samples were analyzed by dark-field microscopy. Morphometric data were acquired with Image J software. Tear volume was positively correlated with both area and time of desiccation. Morphological features of multiple microdesiccates produced from a single subject displayed striking similarities whereas tear microdesiccates from different healthy subjects displayed consistent differences but shared a common general design. This design may be mostly represented by the occurrence of four distinctive zones, named as zones I, II, III and Transition band. The main features of these zones are described.

Key terms: Tear, Tear ferning test, morphometry, dark-field microscopy.

INTRODUCTION

Fern-like crystalloids are normally formed when microvolumes of certain body fluids such as cervical fluid, saliva and tears are allowed to dry spontaneously on a glass surface. Ferning, that is, formation of these crystalloid structures, has been proposed as a valuable tool for the assessment of certain physiological or physiopathological conditions in mammals, including man. Formation of fern-like crystalloids from cervical fluid, the best-known example of this phenomenon, has long been used to determine whether amniotic membranes are leaking and to monitor fertility in humans (Thomasino et al., 2013). Also, reduced ferning has been most frequently described for tear fluid collected from Dry Eye patients compared to normal subjects (Rolando, 1984; Srinivasan et al., 2007; Tabbara and Okumoto, 1982). Furthermore, ever since a four-level grading system (I through IV) was proposed, a Tear Ferning Test (TFT) has been increasingly used to characterize tear fluid during the assessment of patients suffering from dry eyes (Rolando, 1984; Rolando et al., 1988; Albach et al., 1994; Maragou et al., 1996; Cennamo et al., 2008). TFT has a sensitivity in the range from 82 to 94% and a specificity in the range from 75 to 92% (Rolando et al., 1986; Albach et al., 1994; Norn, 1994; Rolando, 2007). Despite its potential to serve as an ancillary diagnostic tool for Dry Eye, several intrinsic and extrinsic limitations of the procedure have precluded it's even wider adoption. A limited consistency in the interpretation and classification of similar TFT images by different authors (Felberg et al., 2008; Beden et al., 2008), a marked influence of environmental factors (temperature and humidity) on the

quality of the fern-like crystalloids (Horwath et al., 2001) and a usual grouping of stages I + II (in support of normality) and III + IV (in support of Dry Eye) (Felberg et al., 2008; Iester et al., 2000) are current limitations of this assessment . Most importantly, a large number of studies using the TFT have documented their observations by displaying images of just a minor fraction of the desiccated tear sample in order to remark on either the representative presence or the representative absence of fern-like crystalloids (Rolando, 1984; Li et al., 2007). Thus apart from conveying some degree of subjectivity, this methodological selection necessarily restricts the information provided by the whole desiccated tear specimen. The aim of this study was to characterize morphological features of desiccated microvolumes of tear samples from healthy donors ("tear microdesiccates"). Microvolumes of tear samples were allowed to dry on standard glass surfaces under ambient conditions. Description will consider the entire desiccated tear material and not just the eventual occurrence of tear ferns.

MATERIALS AND METHODS

Subjects

A total of twenty-three healthy subjects (10 men and 13 women, average age 17.9 ± 1.4 years, age range 17-20 years) who were classmates attending the first course at the University were included in this study. All the individuals fulfilled the following criteria: a) Normal visual parameters, b) Normal tear production as assessed by the Schirmer I test with a reference value of 10 mm at 5 min (van Bijsterveld, 1969), c) Normal tear film stability as determined by the Fluorescein

break-up time (FBUT) test with a reference value of 10 seconds (Vitali et al., 1994), d) Absence of Dry Eye symptoms as defined by an Ocular Surface Disease Index (OSDI) score below 15 (Schiffman et al., 2000) and e) No medication during the last three months (Bron et al., 2007). These subjects acted as tear donor volunteers and signed an Informed Consent. The study was conducted according to the recommendations of the Declaration of Helsinki and approved by both the Ethics Committee of the Faculty of Medicine, University of Chile and the Ethics Committee of Fondecyt (Fondo de Desarrollo Científico y Tecnológico)-Chile.

Tear collection

Tear fluid was collected using polyurethane mini sponges, as reported elsewhere (López-Cisternas et al., 2006). Samples were taken always around 9-11 AM to control for eventual circadian variations. For each eye a single 3-minute tear sample was taken. The amount of sample was determined by gravimetry. Aliquots of the tear sample were taken for desiccation assays and the rest of the sample was stored at – 82 ºC for further analyses.

Tear desiccation and image capture

Unless otherwise specified, duplicate one-microliter aliquots of a fresh sample of tear fluid were taken with a 2-µL Gilson micropipette fitted with an ultrafine tip and placed sharply on a point of a glass microscope slide that had been positioned horizontally. Both tear aliquots were allowed to dry spontaneously at an altitude of 520 m (Santiago, Chile) under ambient conditions of temperature (range 15-25 ºC) and relative humidity (range 40-45%)). Micrographs of the dry samples were taken under a dark-field microscope (Zeiss, Axiostar Plus, objective lens = 5X, ocular lens = 10X) fitted with a Canon Powershot G10 14.7 megapixel digital camera. Fern images were routinely classified by two independent trained operators as types I through IV according to Rolando's criteria (Rolando et al., 1984). Morphometric data of the image corresponding to

each entire desiccated tear sample was acquired with Image J image analysis software (Rasband, 2011).

Materials

Ultrafine gel-loading micropipette tips (Natural round Microflex TM, round capillary section, OD 0.57 mm) were acquired from Therapak Pharma Services Ltd. (Hayes, Middlesex, United Kingdom). Glass microscope slides were obtained from W. Knittel Glass (Braunschweig, Germany). Mini-sponges for tear collection (Pele Tim 0) were purchased from VOCO GmbH (Cuxhaven, Germany).

Statistical analysis

Differences between groups were determined using a two-tailed Student's t test with $p < 0.05$ as the statistical rejection criterion (α).

RESULTS

Volume of tear fluid subjected to desiccation

Tear desiccates produced from aliquots of 1-3 µL of tear fluid taken from individual subjects could be observed integrally under low magnification microscopy (Figure 1). Volumes of tear fluid over 3 µL produced areas of desiccation greater than the field of the microscope. Thus in this study the morphological analysis of single desiccates was mostly focused on images produced after desiccation of 1 or 2 µL of tear fluid. Under these experimental conditions full desiccation of each 1 µL aliquot usually took less than 10 minutes. Morphometric analysis of dark-field micrographs showed that the desiccated tear fluid occupied a roughly circular area, that is, the major diameter to minor diameter ratio of the image was close to 1. The entire area of each desiccated 1 µL-aliquot of tear fluid taken from single healthy subjects was 6.69 ± 0.68 mm^2 (n=23). A linear relationship between volume of tear sample in the range 0.25-2 µL and area of desiccation
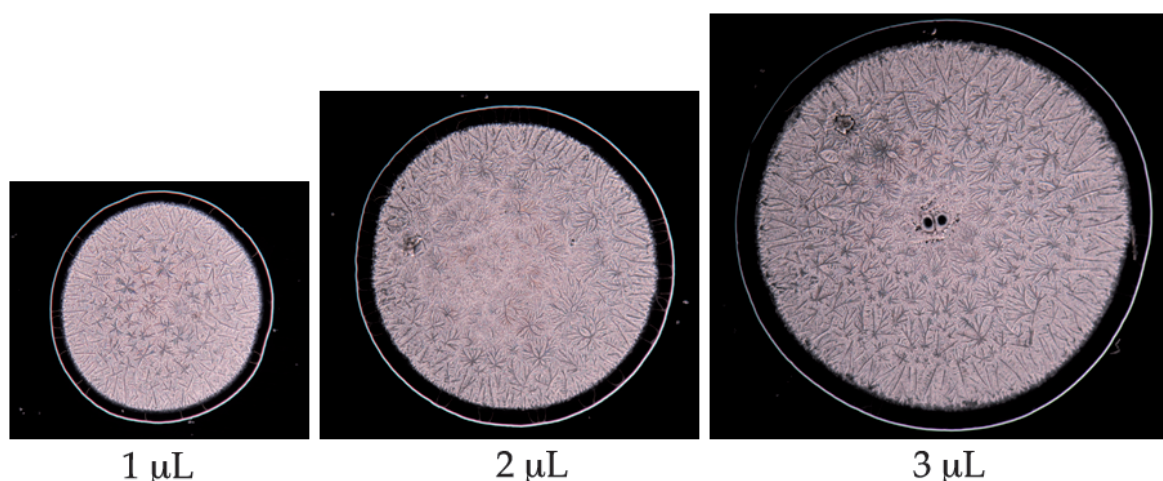

Figure 1. Volume-independence of the morphological appearance of tear microdesiccates. Each of the microdesiccates produced from aliquots of 1 (left), 2 (center) and 3 (right) microliters of a single tear fluid sample displayed identical morphologies. All three images in this representative series are presented at the same magnification. Diameters of the corresponding microdesiccates were 2.86, 4.01 and 5.20 mm, respectively. Further quantitative descriptive data on the microdesiccates of this figure are presented in Table II.

was observed. In addition, a positive non-linear relationship between tear volume in the same range and the time necessary for complete desiccation was also observed (Figure 2). Two independent operators working with the same tear samples obtained similar results (Student's t test p > 0.05).

Constancy and variability in the morphological appearances of tear microdesiccates

Preliminarily, the general morphological appearances of tear desiccates produced simultaneously from aliquots of 1-3 µL of a single sample of tear fluid were compared. In these assays and with no exception we observed that the general morphological appearance of the tear microdesiccates produced from single samples were very similar apart from the total area of desiccation, that is, the morphological appearance of those microdesiccates was volume-independent in the range of volumes under study (see, for instance, Figure 1). Likewise, multiple microdesiccates produced from identical aliquots taken from a single sample of tear fluid showed striking morphological similarities in terms of size, design and shape of structural elements (Figure 3). By contrast, tear microdesiccates produced simultaneously from identical aliquots of tear fluid sampled from different healthy subjects sometimes exhibited marked differences in discrete structural components but shared a common general design (Figure 3).
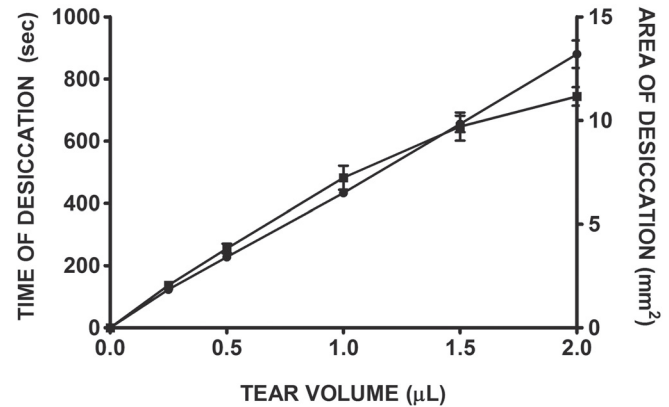

Figure 2. Contact area and time of desiccation of tear microvolumes on a glass surface. Aliquots of 0.25-2 microliters of a single tear fluid of sample placed on a horizontal glass surface were allowed to dry (in triplicate). The time necessary for full desiccation of each aliquot was scored by microscopic inspection. Whole areas of the corresponding tear microdiseccates were assessed by morphometric analysis of images obtained on calibrated microscope observation fields using Image J software. A linear relationship between volume of tear and contact area and a positive nonlinear relationship between volume of tear and desiccation time were observed. The graph is representative of three independent experiments.
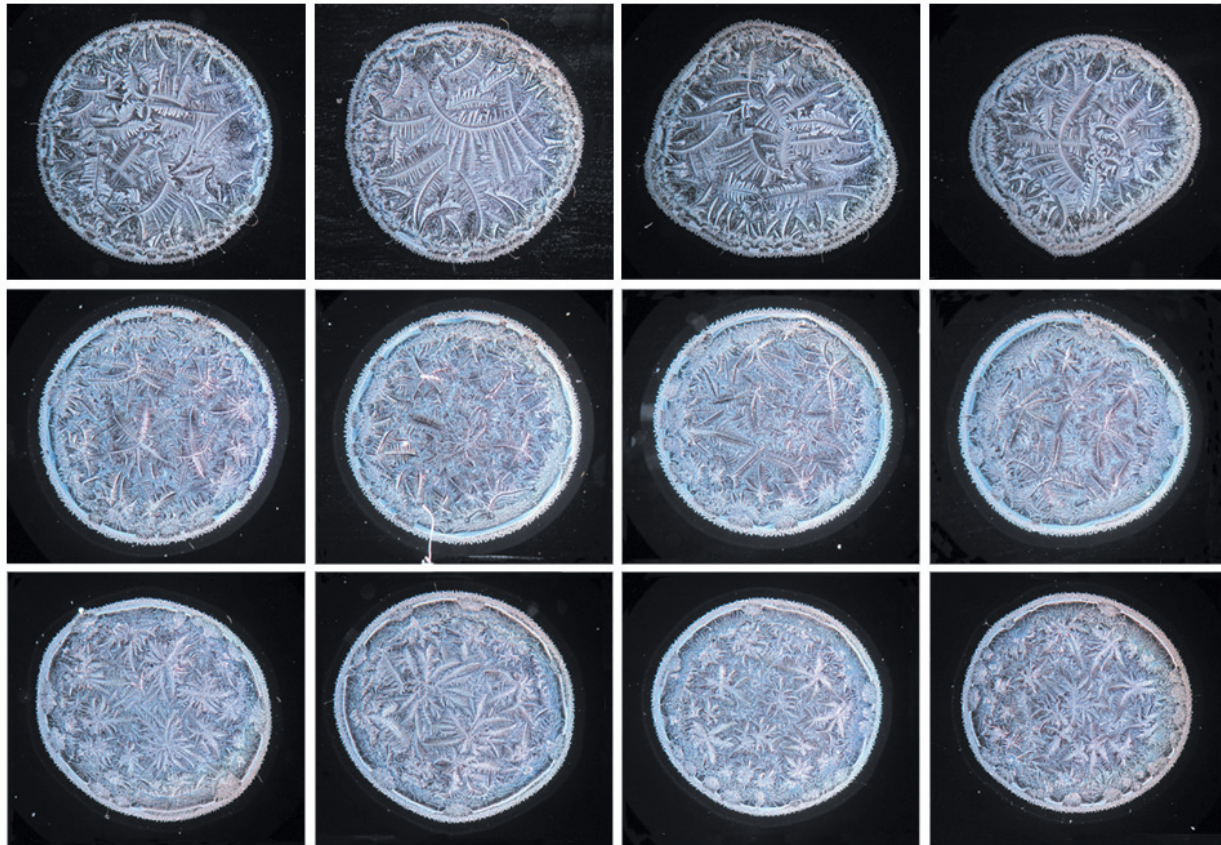

Figure 3. Similarities and differences among tear microdesiccates. Quadruplicate of one-microliter aliquots taken from single samples of tear fluid collected from healthy subjects were desiccated simultaneously on a glass surface. Representative tear microdesiccates from three subjects are shown (rows). Note the marked similarities among tear microdesiccates from each subject together with some also clear differences among the microdesiccates from different subjects. All these microdesiccates were rated I-II (normal) in the conventional Rolando classification system for tear ferning pattern.

Zones in a desiccate of tear fluid

A systematic comparison of microdesiccates from 54 tear samples obtained from right and left eyes of 23 healthy subjects allowed us to identify a general design. A tear microdesiccate consists of four main parts, namely zones I, II and III and a transition band (Figure 4). **Zone I**: The outermost component of the microdesiccate. It consists of a structured hyaline amorphous material that surrounds the whole circular area of desiccated tear fluid. In the group of 54 samples from healthy subjects, the width of zone I represented on the average 6.76 ± 2.59% of the radius of the circular tear desiccate. The wide distribution of this relative width of zone I (range from 2.25 to 14.48%) was due to significant inter-individual differences in this parameter. Thus in a study with 8 randomly chosen healthy subjects we observed that the standard deviations of intra-individual determinations of zone I width were just 5.76% of the corresponding averages. Another major observation in this characterization was that the width of zone I relative to the corresponding radius of the circular tear microdesiccate from a single tear sample was a constant for any tear volume between 1 and 3 μL. For instance, the tear microdesiccates shown in Figure 1 obtained from different volumes of a single tear sample had a relative zone I width of 10.39 ± 0.44%. On the other hand, zone I accounted for an average of about 15% of the entire area of any single microdesiccate of tear collected from a group of healthy subjects (range 5.9-27.1%, n = 23). Again, intra-individual determinations of the relative contribution of zone I to the total area of tear microdesiccates showed standard deviations usually less than 10% of the corresponding averages from triplicate determinations (Table I). Typically, a variable number of transverse refringent fracture-like or crack-like structures could be readily observed in zone I. **Zone II**. Consists of a band of clear-cut crystalloid structures, which appear to emerge centripetally from regularly spaced points in proximity to zone I (Figures 5A and 5B). The two most common structures of this zone are fern-shaped and leaf-shaped crystalloids. In our study, zone II together with the transition band (see below) represented usually over 40% of

the entire area of any single normal tear microdesiccate (Table I). **Zone III**. This domain corresponds to the center of the desiccated tear sample and is characterized by the occurrence of typical fern-like structures displaying a variety of forms
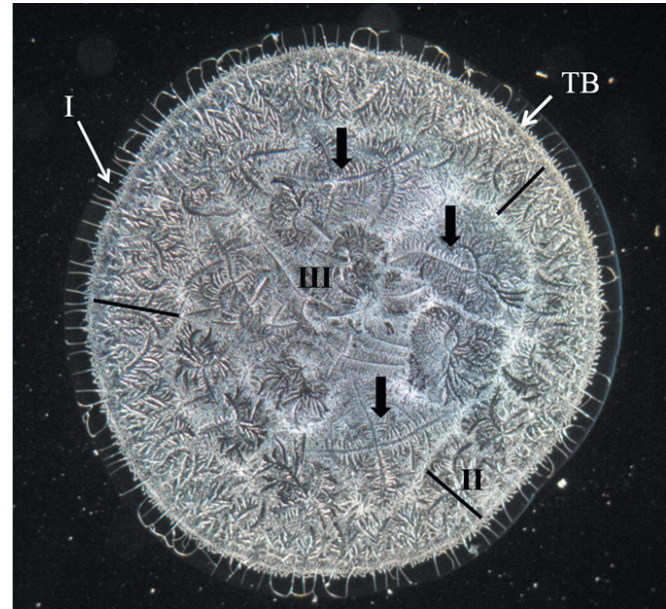

Figure 4. Zones in a normal tear microdesiccate Representative microdesiccate produced from a 1-μL aliquot of tear (2.81 mm in diameter). **a. Zone I**, a hyaline material that surrounds the whole circular area of the tear microdesiccate with a variable number of transverse and highly refringent structures that resemble fractures or cracks, **b. Zone II**, a band (marked with black lines) of clear fern-shaped or leaf-shaped crystalloid structures emerging centripetally from regularly spaced points in proximity to zone I. **c. Zone III**, the center of the desiccated tear sample presents a variety of crystalloid structures differing in robustness, length and branching (black arrows). **d. Transition band (TB)**. Occurring in most of the desiccated tear samples, this morphologically distinct structure is located along the entire interphase between zones I and II.

TABLE 1

Relative contribution of each zone to the total area of normal tear microdesiccates

	Area[a] (mm²)	Zone I[d]	Zone II[cd]	Zone III[d]
Subject 1[b]	7.11 ± 0.68	0.170 ± 0.010	0.407 ± 0.042	0.423 ± 0.052
Subject 2	5.85 ± 0.39	0.152 ± 0.014	0.466 ± 0.029	0.382 ± 0.015
Subject 3	5.79 ± 0.40	0.147 ± 0.002	0.435 ± 0.048	0.418 ± 0.047
Subject 4	6.73 ± 0.51	0.128 ± 0.011	0.359 ± 0.060	0.513 ± 0.070
Subject 5	6.76 ± 0.20	0.178 ± 0.009	0.408 ± 0.031	0.414 ± 0.038
Subject 6	7.38 ± 0.03	0.264 ± 0.008	0.476 ± 0.021	0.260 ±0.021
Subject 7	6.70 ± 0.39	0.117 ± 0.002	0.512 ± 0.046	0.370 ± 0.047
Subject 8	7.44 ± 0.26	0.065 ± 0.008	0.610 ± 0.003	0.325 ± 0.005

a Whole area of microdesiccates produced from 1 μL of tear
b Mean ± standard deviations of three determinations per subject
c Transition band is included
d Zone area to Whole desiccate area ratio
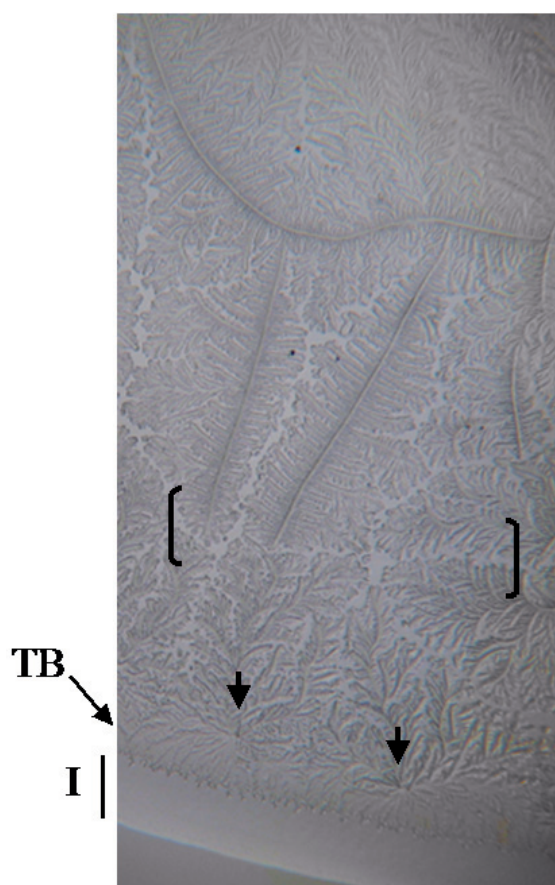
differing from each other in robustness, length and branching. In our study, this zone was also found to represent about 40% (range from 37 to 57%, n = 23) of the entire area of normal tear microdesiccates (Table I). Overall, a few disperse points in this zone seem to serve as origin sites for the multidirectional radial growth of fern-like structures. These structures end where they have come into contact with other crystalloid structures from either zone II or zone III (Figure 5A). Thus a clear-cut boundary between zones II and III could be usually identified (Figure 5A). **Transition band**. This fourth structural element of a normal tear microdesiccate, which was found to be present in most, but not all of the desiccated tear samples, is located along the entire interphase between zone I and zone II (Figures 4, 5A and 5B). Some discrete morphological subtypes of Transition band could be recognized (Figures 6A and 6B). The transition band seems to influence the organization of zone II by providing "anchoring points" for its crystalloids (Figures 5A and 5B). Altogether, the individual contributions of zones I, II and III to the total area occupied by a single tear microdesiccate remained unmodified after desiccating any volume of tear fluid in the range between 1 and 3 µL (Table II).

DISCUSSION

In this study we carried out a morphological characterization of desiccated microliter volumes of tear samples at the light microscope level. Aliquots of tear fluid were desiccated by a standard procedure used in the conventional Tear Ferning Test (Rolando, 1984; Bron et al., 2007). The analysis of each sample was conducted by two independent trained operators and was distinctively addressed not only to the central zone of the image where fern-like microcrystalloids might
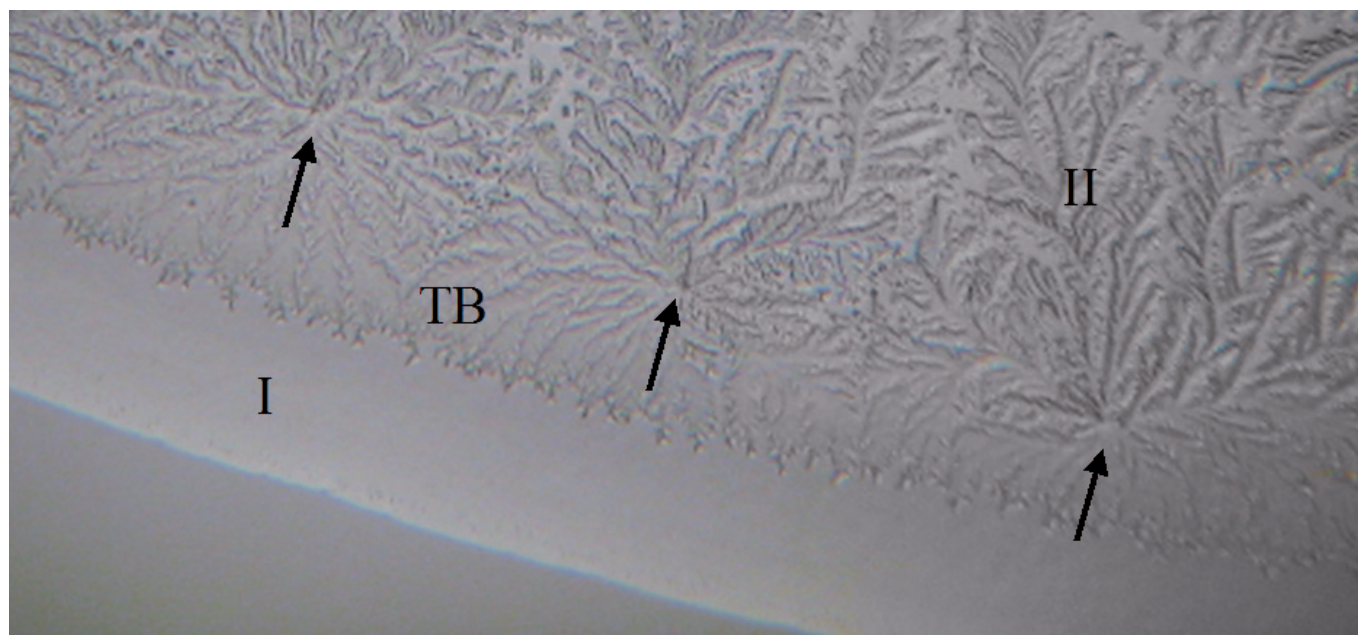

Figure 5. Fine structural details of a tear microdesiccate. A. Fern-like crystalloid structures of zone III end where they have come into contact with other crystalloid structures from either zone III or zone II. Thus a sharp boundary between zones II and III can be identified (brackets). Also, limits between neighbor crystalloids of zone III become readily visible as empty spaces. On the other hand, the transition band (TB) at the zone I-zone II interphase seems to provide anchorage points for zone II crystalloids (arrowheads). **B.** These anchorage points are located at regular intervals on the border between the transition band and zone II. The whole microdesiccate of figures 5A and 5B was 2.71 mm in diameter.

form, which seems to be the usual practice, but to the entire desiccated tear sample (Rolando, 1984; Beden et al., 2008; Li et al., 2007). For convenience, we named these specimens "tear microdesiccates". In microdesiccates of tear fluid collected from healthy subjects we identified four common components that we named Zones I, II, III and Transition band. Major morphological features of each of those components were described.

A tear microdesiccate can be considered a highly reproducible representation of the components occurring in a particular tear sample. In support of that view, we have now shown that multiple microdesiccates produced from a single tear sample display striking similarities in their fine morphological features. In this study we also showed that tear microdesiccates from different healthy subjects may display clearly different fine morphological features. Thus, each tear microdesiccate would not be the end result of a stochastic phenomenon but its formation appears to be the expression of the exclusive physicochemical properties of the corresponding tear fluid. Accordingly, the identification of morphological parameters in tear microdesiccates may well be of value for comparison purposes, for instance between tears from healthy donors or between tears across different health conditions. This kind of comparison would necessarily demand the identification of morphological features that are common to tear samples from healthy subjects. The four components or zones occurring in a normal roughly circular and symmetric tear microdesiccate seem to satisfy this condition.
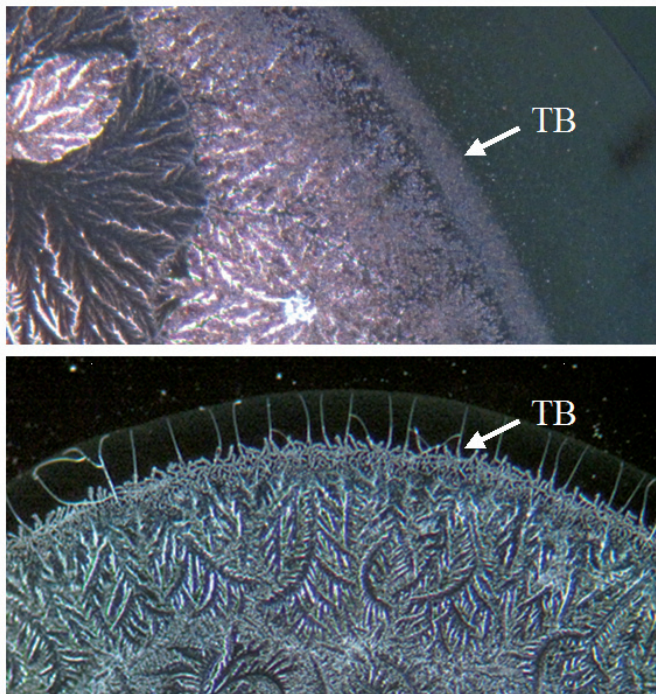

Figure 6. Variations in the transition band. About two thirds of the tear samples from healthy subjects in this study displayed noticeable transition bands. Some morphological variations in this domain of the tear microdesiccates could be appreciated. The most common types of transition bands were **A.** Compact (top) and **B.** Filamentous (bottom). Actual sizes of the tear microdesiccates were 2.81 and 2.90 mm diameter, respectively.

Formation of this four-zone structure must be a complex process, as is the desiccation of any sessile drop of a colloidal dispersion (e.g. tear) on a glass surface (Sharfrin and Zisman,1960). Initially the contact line, that is the tear-glass-air interphase, would become fixed and the drop would maintain a fixed tear-glass contact area. Considering that not all colloidal dispersions become fixed to the glass surface during their desiccation, the macromolecular composition of tear fluid (mucins and proteins) would play a crucial role in this property of the tear fluid. Subsequently, suspended particles (some tear components) would be deposited in a ring at the periphery of the tear drop (Zone I of the microdesiccate) due to capillary flow, while a crust is formed on the free surface of the drop due to evaporation. The crust would evolve through different shapes as the tear-glass contact area remains constant and the tear drop volume decreases due to water evaporation. The crust would eventually collapse to generate zones II and III of the tear microdesiccates (adapted from Duggal et al., 2006). Thus the spatial distribution of the tear components occurring in a sessile drop of tear fluid placed on a glass surface may be critical for the organization of tear crystalloids. Regularly-spaced anchoring sites on the transition band serving as origins for Zone II crystalloids could represent evidence of a highly organized supramolecular organization of the tear fluid components, as is implicit in the tear film bilayer or trilayer models (Braun, 2012). However, it is not yet known to what extent this distribution reproduces *in vitro* the molecular organization of the tear film. As with the fern-like crystalloids, the ions, molecules, macromolecules or the interactions among them that underlie the whole organization of the tear desiccates are also mostly unknown. In this respect, some authors have suggested that relative concentrations of either tear mucin (Rolando, 1984) or tear ionic components (Kogbe et al., 1991) are crucial for the organization of fern-like crystalloids. Thus, a foreseeable challenge to meet in the future will be to localize somewhere in the multidomain structure of the tear desiccates a number of well-known components of the whole tear fluid, such as tear, mucins and several major proteins (Ohashi et al., 2003). Studies in this regard are necessary to obtain insights into the ability of some or all the tear components to interact with each other, thus producing the artificial supramolecular organization of the tear desiccates, and also to learn about the interaction properties

TABLE 2

Volume-independence of the relative contribution of each zone in a normal tear microdesiccate

	Volume of Tear Fluid			Mean (± SD)b
	1 μl[a]	2 μl[a]	3 μl[a]	
ZONE I	0.206	0.210	0.199	0.205 ± 0.006
ZONE II[C]	0.466	0.553	0.514	0.511 ± 0.044
ZONE III	0.328	0.237	0.288	0.284 ± 0.046

a Whole areas of the tear desiccates shown in Figure 1: 6.4, 12.6 and 21.2 mm[2], respectively
b Mean ± standard deviation of three independent determinations on a single tear sample
c Transition band is included
d Relative contribution = Zone area to Whole desiccate area ratio

of tear molecules that *in vivo* are necessarily responsible for tear film stability. In this respect, a reduced ability of tear components to organize fern-like structures (Rolando, 1984; Tabbara and Okumoto, 1982; Maragou et al., 1996; Cennamo et al., 2008) as well as changes in the biochemical composition of the tear fluid (Ohashi et al., 2003; Grus et al., 2005; Versura et al., 2010; Boehm et al., 2011) have been recurrently associated with Dry Eye symptoms. Thus, based on the information included in this report it would be expected that side-by-side comparison of the four-zone morphology of tear microdesiccates (or its alterations) and molecular data from the corresponding tear fluid in patients with specific diseases, such as evaporative Dry Eye and aqueous deficient Dry Eye, may help find more specific correlates (Behrens et al., 2006; Dogru et al., 2005; Gumus and Cavanagh, 2009; Lemp et al., 2007; Matoba et al., 2003; Tseng, 2011). Accordingly, architectural and molecular analysis of microdesiccates from human biofluid films may become a novel and productive way to assemble differential diagnosis and patient-oriented therapeutic management, particularly when biofluid sampling itself is a delicate task.

ACKNOWLEDGEMENTS

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