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Bottom–up protein identifications from microliter quantities of individual human tear samples. Important steps towards clinical relevance.

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ABSTRACT

A relatively simple combination of Schirmer strip sampling with straightforward sensitive nanoLC quadrupole-Orbitrap tandem mass spectrometry after a minimum of sample processing steps allows for replicate proteomic analysis of single human tears, i.e., without the requirement for sample pooling. This opens the way to clinical applications of the analytical workflow, e.g., to monitor disease progression or treatment efficacy within individual patients. Proof of concept is provided by triplicate analyses of a singular sampling of tears of a dry eye patient, before and one and two months after minor salivary gland transplantation. To facilitate comparison with the outcome of previously reported analytical protocols, we also include the data from a typical healthy young adult tear sample as obtained by our streamlined method.

With 375 confidently identified proteins in the healthy adult tear, the obtained results are comprehensive and in large agreement with previously published observations on pooled samples of multiple patients. We conclude that, to a limited extent, bottom–up tear protein identifications from individual patients may have clinical relevance.

1. Introduction

Since the recent launch, within the Human Proteome Organization (HUPO), of the Human Eye Proteome Project (HEPP) [10,8], tears are among the body fluids which have gained increasing interest as a source of diagnostic markers not only for ophthalmological diseases, but also for systemic and neurological disorders. Whereas accounts of proteins identified from human tears all resulted from the analyses of pooled samples (see advanced LC MS/MS reports by de Souza et al. [3]; Zhou et al. [15]; Srinivasan et al. [12]; Salvisberg et al. [11]; and the references quoted therein to other earlier (and generally less performant) mass spectrometry based proteomics approaches), we focus on what can be achieved by studying individual tear samples with the latest LC MS/MS.

In order to be compliant with the envisioned clinical application, we compiled an efficient analytical workflow with minimal sample preparation steps.

We opted for Schirmer strips (instead of capillaries) as most convenient clinician friendly tear sampling tools. On these filter paper strips >20 μl volumes of tear can be easily collected. This minimally invasive form of body fluid collection is highly accepted in the primary healthcare setting and has great potential for use in health screening [9]. As such it is already common use in current ophthalmological practice, e.g., for testing the severity of dry eye disease.

Employing straightforward nanoLC tandem MS by a recently introduced high resolution quadrupole-Orbitrap hybrid system, we demonstrate that it is realistically feasible to perform multiple replicate proteomic analyses (in terms of bottom–up protein identifications) on these microliter sample quantities.

As such the overall sensitivity of this optimized analytical protocol permits intra-individual (unpooled) monitoring of e.g., disease progression or treatment, with several hundreds of relevant data points (protein identifications and relative quantitations) collected for each clinical sample.

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As proof-of-concept we monitored tears of a severe case of keratoconjunctivitis sicca before and after surgical treatment. Keratoconjunctivitis sicca, or dry eye syndrome is a very complex multifactorial disease [7], which, as the name indicates, in virtually all cases results in reduced tear volume production, which is reported to be associated with a decreased general lacrimal protein secretion. Very severe cases are uniquely treated by autotransplantation of a minor salivary gland into the eye, a technique originally introduced by Prof. J. Murube and perfectionized over the past ten years [5]. The rationale behind a proteomics analysis of clinically sampled tears is that comparative protein composition analysis of tears from diseased versus treated and/or healthy eyes, may yield medically relevant information regarding both the effectiveness of the treatment and the possible disease etiology.

2. Materials and methods

2.1. Reagents and chemicals

Ammonium bicarbonate, *tris*(2-carboxyethyl)phosphine (TCEP), iodoacetamide, formic acid, dimethyl sulphoxide, as well as trypsin were from Sigma–Aldrich™.

2.2. Tear collection and sample preparation

Human lacrimal fluids were sampled using Schirmer tear test strips (Haag-Streit, UK), principally as published earlier by Zhou et al. [15] and Srinivasan et al. [12]. For this the paper strip was tenderly placed inside the lower eyelid, after which the subject was instructed to gently close the eye. The moistened strip was removed after a maximum of 5 min. The sampling procedure did not include any anesthetizing eye drops. Both during sampling as well as further strip handling, gloves were worn.

Two different individuals provided the tear samples used in this study (Table 1). Tear samples from an individual diagnosed with severe dry eye syndrome (aqueous deficiency subtype) were collected (with the patient's consent) at 3 different time points during disease treatment, i.e., before treatment, and 1, and 2 months after surgery (minor salivary gland transplantation). Consistent with the data in the literature [12] the aqueous deficient dry eye typically scored <5 mm of Schirmer strip wetting. For comparative purposes one additional tear sampling of a healthy young adult male volunteer was included in this method evaluation study. Healthy adult tears have been consistently analyzed by the relevant proteomics methods described in the literature [3,15,12,11]. The healthy tear easily moistened >15 mm during sampling.

After sampling, strips were stored in labeled protease-free Eppendorf vials at −20 °C until further analysis. For analysis 2 mm of the wetted part of the filter paper area which had not been in direct contact with the eye ball and conjunctiva (in order to minimize sample contamination with epithelial proteins) was carefully cut from each strip. During sample processing, care was taken to keep the analysis volume to an absolute minimum, to remain maximally compatible with the limited nanoLC injection volume. After transfer to another protease-free microcentrifuge tube the 2 mm ribbon was carefully cut into minute equally sized pieces using clean scissors. The resulting shreds were submerged in 47 μl of 25 mM ammonium bicarbonate (pH 8.0) for 90 min. Reduction of disulfide bonds was achieved by mixing 1 μl of TCEP (×50 stock solution; 10 mM final) with the sample for 30 min. Subsequent alkylation was allowed to occur for 45 min after addition of 1 μl iodoacetamide (×50 stock solution; 20 mM final). Finally overnight protein digestion (RT) was initiated by adding trypsin (sequencing grade; 1 μl of 200 ng/μl stock). Afterwards 5 μl of a mixture of 5% DMSO and 5% formic acid were added to assist resuspension of tryptic peptides.

Table 1
Tear sample donor list (same color row indicates that sample originated from the same individual).

Sample code	R/L	Sex	Age	Clinical origin
Y	R	M	26	Healthy volunteer
59	R	M	69	Dry eye patient (untreated eye)
60	L	M	69	Dry eye patient (treated eye, 1 month after surgery)
61	L	M	69	Dry eye patient (treated eye, 2 months after surgery)

Table 2
Gradient elution profile (A: Milli Q; B: 80% acetonitrile, 0.1% formic acid).

Time (min)	Flow (nl/min)	% A	% B
0	350	98	2
40	350	70	30
57	350	35	65
59	350	0	100
60	350	0	100

2.3. Sample analysis

Of each sample 5% of the total reaction volume (2.5 μl) were analyzed by Easy-nLC 1000™ ultra performance liquid chromatography on a 200 mm long in-house packed C18 nano HPLC column (50 μm ID). A 60 min elution gradient (solvent B: 80% acetonitrile, 0.1% formic acid; solvent A: Milli Q; 350 nl/min) was applied as detailed in Table 2.

Tandem MS analysis was carried out on a Q Exactive Plus™ quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptide fragmentation was by high energy collision induced dissociation (HCD), with the MS^2 settings are summarized in Table 3.

2.4. Data analysis

Spectral files generated (Xcalibur™, RAW format) were analyzed using Proteome Discoverer™ software version 1.4. Multiply charged peptide spectra were deconvoluted to singly charged spectra and deisotoped. The spectral files were then searched against the Uniprot *Homo sapiens* reference proteome (UP000005640; release Oct, 2014) using the Sequest™ HT algorithm (Thermo Fisher Scientific; parameters see Table 4).

Table 3
Full scan MS and data dependent MS/MS settings of the Q Exactive Plus™ system. Abbreviations: AGC, automatic gain control; dd, data dependent; IT, injection time; NCE, normalized collision energy.

Properties of full scan/dd-MS^2	
Resolution full MS	70,000
AGC target full MS	3e6
Maximum IT	250 ms
Scan range	300-1400 *m/z*
Loop count	10
dd resolution	15,000
dd target	1e5
dd-MS^2 max IT	150 ms
Isolation window	2.5 *m/z*
Fixed first mass	100.0 m/z
NCE	28
dd underfill ratio	0.5%
Charge exclusion	Unassigned, 1, >8
Peptide match	Preferred
Dynamic exclusion	30 s