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TECHNICAL BRIEF: Optimized pipeline for isolation of high-quality RNA from corneal cell subpopulations

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Purpose: Attempts to determine the transcriptional profile of discrete subsets of limbal epithelial cells in situ using laser capture microdissection (LCM) face two major challenges. First, the transcriptional profile of cells within a tissue may rapidly change as the tissue is excised and exposed to cold ischemia. Second, there is a risk of degradation of the RNA as the cellular compartment is separated from the remaining tissue. An optimized protocol for LCM of corneal epithelium is presented to address these issues.

Methods: Experiments using porcine eye globes were carried out to determine both optimal procedures and settings for tissue harvest, transport, storage, histology, LCM, and RNA isolation. The optimized protocol was validated using human corneal epithelium.

Results: To facilitate preservation of the gene expression profile, we have developed a mechanical tool for dissection of cornea that, in combination with flash freezing, enables tissue to be stored within 5 min of enucleation of the eye. Furthermore, we describe how RNA from limbal crypt cells may be obtained using a procedure involving cryosectioning, histological staining, and LCM.

Conclusion: In this paper, we describe an optimized method for isolating high-quality RNA from cellular subpopulations confined to the limbal crypts of the cornea. The procedure yields RNA in amounts and quality suitable for downstream gene expression analyses, such as microarrays or next generation sequencing.

The corneal epithelium is continuously degraded by wear and tear, and therefore depends on regeneration by a rare population of unipotent stem cells in the corneoscleral limbus termed limbal epithelial stem cells (LESCs). LESCs divide and mature as they migrate toward the more central and superficial areas of the cornea [1,2]. In conditions where the LESC population becomes compromised, conjunctival tissue migrates onto the cornea, resulting in decreased visual acuity and pain. Several strategies for treating LESC deficiency by transplanting expanded populations of limbal epithelial cell cultures containing smaller or larger proportions of LESCs have been devised [3]. However, to fully harness the LESC regenerative potential for clinical purposes, better molecular characterization of the stem cell compartment in situ is necessary [4].

To characterize a small subpopulation of cells within a tissue, laser capture microdissection (LCM) appears optimal [5,6]. In this procedure, well-defined regions of cells are excised from complex tissue structures, after which the mRNA from the cells of interest can be isolated and analyzed with either microarrays or sequencing. For this procedure to be effective, rapid harvest of RNA is imperative to avoid RNA degradation due to endogenous and exogenous endonucleases. Furthermore, the tissue should be treated in a way that the global transcriptional profiles of the different cells are not changed due to cold ischemia, for example, as could be the case when dealing with cadaveric tissue. Specifically, a study of the effect of ischemia on gene expression in resected colon tissue showed that within 30 min after surgery, the relative levels of 20% of the transcripts had changed [7]. Thus, ideally, when attempting to unravel the transcriptional signature of cells in living tissues, it should be processed immediately upon harvest. This presents a challenge that when working with fresh human ocular tissue, which is removed in the operating theater, the tissue should be processed using tools that are brought into either the operating theater or adjacent rooms. Furthermore, all following steps, including transport, storage, histology, LCM, and RNA extraction, should support maintenance of RNA integrity.

In this paper, we describe a method for isolating mRNA from cells residing in limbal crypts. The isolation is performed in a manner that preserves the intact mRNA expression profiles through rapid dissection of the tissue in the operation theater using a novel guillotine-type cutting apparatus. With this dissection method, tissue can be flash-frozen within 5 min of eye removal. Furthermore, this paper details the transport of the tissue, cryosectioning, and staining

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under RNA-preserving conditions, isolation of cells residing in the limbal crypts by LCM, and finally, mRNA isolation from these cell populations. This pipeline has been validated in experiments yielding deep transcriptomic analysis of human limbal niche compartments [8].

METHODS

Protection against endogenous nucleases: Throughout the entire procedure, all work surfaces and instruments were cleaned in 70% ethanol followed by the RNase AWAY (VWR-Bie & Berntsen, Herlev, Denmark) treatment according to the manufacturer's protocol. All glassware and forceps were heat sterilized for 4 h before the experiments were initiated. Furthermore, staining and storage were performed in certified RNase-free pap jars (Evergreen Scientific, Los Angeles, CA). To avoid contamination with exogenous RNases from saliva and skin, protective clothing including masks, hair nets, and sterile gloves were used. Only certified RNase-free epT.I.P.S. Dualfilter pipette tips were used throughout the experimental protocol (Eppendorf, Hauppage, NY).

Tissue procurement: Porcine eye globes were acquired from the veterinary facilities at Aarhus and Aalborg University Hospitals according to national guidelines and conforming to the standards of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Animals underwent general anesthesia during surgery using a mixture of Zooletil 50 Vet. (25 mg/ml Tiletamine and 25 mg/ml Zolazepam; Virbac, Kolding, Denmark), 2.5 ml Torbugesic Vet. (10 mg/ml Butorphanol; Scanvet, Fredensborg, Denmark), 6.25 ml Rompun Vet. (20 mg/ml Xylazine and 1.5 mg/ml Methylparahydroxybenzoate; Bayer HealthCare, Copenhagen, Denmark) and 1.25 ml Ketamine (100 mg/ml; Intervet, Ballerup, Denmark). This solution was administered as an intramuscular injection with 2.5 ml solution per kg animal. During surgery Fentanyle-Hameln (50 µg/ml; Hameln Pharma Plus, Hameln, Germany) and Midacolam Hameln (5 mg/ml; Hameln Pharma Plus) were used as analgesics. Immediately after surgery, animals were euthanized using intravenous injection of 20 ml Pentobarbital (300 mg/ml) (Skanderborg Pharmacy, Skanderborg, Denmark). Handling of animals was carried out by trained veterinary assistants. Human material was procured with written and informed consent, and with approval from the North Denmark Region Committee on Health Research Ethics. Human bulbus was enucleated by a trained surgeon and handed over in the surgery room. All tissues were processed immediately after surgery. For each harvest, the eyeball was cleaned, the frontal part dissected, and the iris removed, leaving corneal tissue surrounded by the limbus and an outer ring of conjunctival tissue (Figure 1A). This tissue was placed in the corneal slicer (for detailed drawings, please refer to Figure 2), where the tissue was rapidly divided into parallel sections (Figure 1B,C).

We tested two methods for freezing the tissue sections. Either the resulting pieces were immediately oriented in Tissue-Tek OCT compound (Sakura Finetek, Alphen aan den Rijn, the Netherlands) inside cryomolds (Sakura Finetek) and flash-frozen in a combination of isopenthane and dry ice before being transported on dry ice to the laboratory or the sections were stored in RNAlater (Sigma-Aldrich, St. Louis, MO) and transported at room temperature to the laboratory for subsequent flash-freezing. The frozen tissue blocks were stored at −140 °C until further processing.

Cryosectioning and histological staining: Metal-framed polyethylene naphtalate (PEN) membrane slides (Applied Biosystems, Life Technologies, Naerum, Denmark) were initially irradiated by ultraviolet (UV) light exposure with 3,000 µJ/cm^2 using a Stratalinker UV Crosslinker 2400 (Agilent Technologies, Santa Clara, CA) for 30 min. The UV irradiation enabled better mounting of cryosections and sterilized the slides. The frozen corneal tissue blocks were mounted inside the cryostat (Microm Cryostat type HM 505N; Microm International GmbH, Germany), and left for 30 min for the temperature to equilibrate before sectioning. The tissue blocks were then sectioned so that the corneal plane was perpendicular to that of the cutting plane (Figure 1C). For the cryosectioning of corneal tissue, the optimal parameters were determined empirically to be a cutting angle of 11°, cutting temperature −30 °C, and cutting thickness of 10 µm. The precise parallel divisions of tissue samples by the corneal slicer provided good alignment of the tissue surface and the knife-cutting plane thus allowing for serial sectioning of the entire corneal area with only a minimal loss of the sample due to trimming. Phase contrast microscopy was next used to identify slices that featured limbal crypts, and the preparations were subjected to histologic procedures, described in detail below, to prepare for LMC.

Cryosections of interest were mounted on irradiated PEN membrane slides and immediately placed in ice-cold 70% ethanol inside the cryostat for 2 min for initial fixation followed by staining either with the HistoGene LCM Frozen Section Staining Kit (Applied Biosystems) according to the manufacturer's instructions or with cresyl violet by a modification of the method described by Bevilacqua et al. [9]. Briefly, fixed slides were washed with five dips in 4 °C precooled RNase-free water and stained for 10–20 s by pipetting 100 µl of ice-cold cresyl violet solution (0.01 mg cresyl violet/1 ml absolute ethanol) directly onto sections. Excess stain was poured off, and the slides were placed back into the

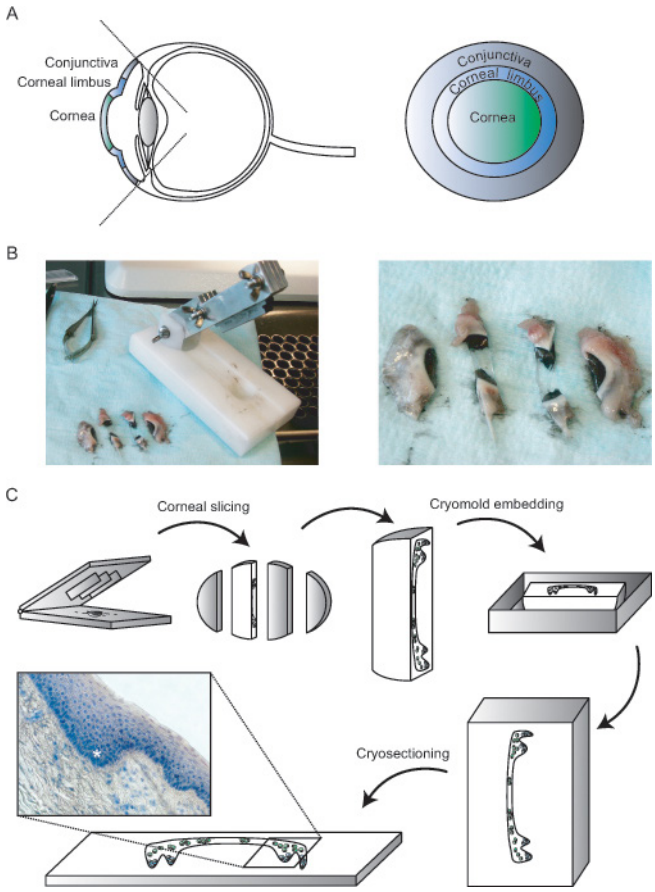

Figure 1. Preparation of histological sections from cornea. **A**: The dotted lines in the left panel represent the line of dissection of the bulbus, and the dissected eye segment is shown on the right. **B**: The corneal slicer was constructed to feature a Teflon base with a spherical depression, into which the cornea was placed face down, and the metal frame into which three parallel blades were fixed 4 mm apart. The resulting corneal blocks are shown on the right. **C**: Schematic outline of the procedure, including a detail of the corneal limbus from slice stained with cresyl violet. The asterisk indicates a limbal crypt.

ice-cold 70% ethanol for 30 s followed by drying in 100% ethanol for 2 min. The staining procedure was finalized in ice-cold xylene for 5 min followed by immediate transportation to the LCM facility using RNase-free pap jars. Slides were processed within 60 min. An example of a stained cryosection is shown in Figure 1C.

Laser capture microdissection: First, the tissue sections were sandwiched between the PEN membrane slide and a supporting glass slide and placed in the Arcturus Veritas (model 704; Arcturus Bioscience, Mountain View, CA) equipped with an infrared (IR) capture laser (150 mW, maximum output at 804–813 nm) and a UV cutting laser (1W, 250 µJ, 15 ns pulse, maximum output at 349 nm) for LCM (Figure 3A). Then, after the limbal crypts were identified (Figure 3B), the thermoplastic ethylene vinyl acetate (EVA) membrane of the cap was melted and glued with the PEN membrane specifically over the area of interest by applying IR laser pulses, and the targeted samples were released from the remainder of the tissue and the PEN membrane by cutting with the UV laser (Figure 3C). The caps were then lifted, and following microscopic verification for the efficiency of the procedure, the captured cells were processed for the RNA (Figure 3D). To estimate the effect of the LCM procedure on the RNA integrity, parallel stained cryosections of tissue were scraped off slides with a scalpel and processed alongside the tissue harvested by LCM.

RNA isolation, quantitation, and quality control: The thermoplastic film containing captured material was stripped off from the caps with sterile forceps and placed in 0.5 ml RNase-free tubes containing 50 µl extraction buffer from the PicoPure RNA isolation kit (Applied Biosystems). Total RNA isolation was performed according to the manufacturer's protocol, and included an additional step of DNase I incubation (Sigma-Aldrich). Total RNA was eluted in 11 µl of elution

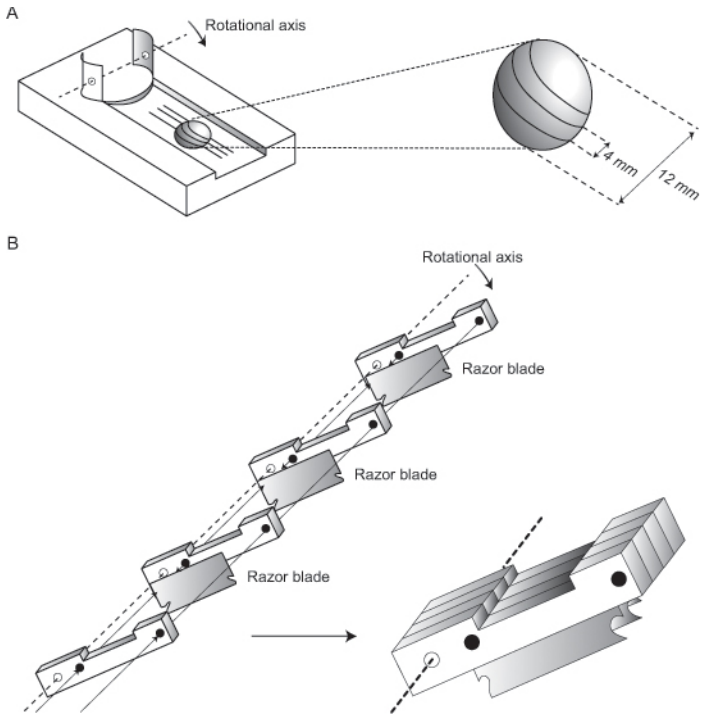

Figure 2. Design of corneal slicer. **A**: A Teflon base was constructed with a central pit measuring 12 mm in diameter designed to fit a cornea. The base also has three parallel grooves to accommodate razor blades from the cutting arm. **B**: The cutting arm consists of four stainless steel blocks separating three disposable razor blades by 3.9 mm. All elements are fixed in place with a pair of clamp bolts (closed circles), and the entire assembly is attached to the base at the point of the rotational axis with another clamp bolt (open circles). All components can be sterilized in an autoclave. The dashed line indicates the rotational axis for the cutting arm.

buffer and immediately stored in at –140 °C. The integrity and the concentration of the total RNA were measured using the Agilent Bioanalyzer 2100 system (Figure 3D) with the Agilent RNA 6000 Pico Kit system. The RNA integrity was determined by assigning an RNA integrity number (RIN number), where a value of 10 denotes intact RNA and a value of 1 indicates complete degradation [10].

RESULTS AND DISCUSSION

Tissue harvest and freezing: For transcriptional profiling studies, great consideration must be given to the speed with which the tissue is processed, as changes in the mRNA levels may occur within minutes [7]. In a comparison of either flash-freezing the tissue immediately upon sectioning or after hours of storage in RNA later, we found storing tissues in RNA later introduced gross disturbances in the tissue architecture, rendering it virtually impossible to distinguish the crypt structures after staining. We thus recommend flash-freezing corneal tissue blocks immediately upon removal, resulting in a procedure where corneal tissue may be harvested, sectioned, and flash-frozen within 5 min of the enucleation of the eye to maintain expression profiles. Furthermore, we recommend the use of the tissue slicer, which, in addition to rapid tissue processing, provided the additional benefit of generating tissue blocks with parallel cut edges, such that when the tissue blocks were placed inside the cryomolds, the top of the tissue block was parallel to the cutting plane. This maximized the yield of corneal tissue sections suitable for laser microdissection.

Preparation of histological sections for laser capture microdissection: During the 30 min of temperature equilibration, the cryostat should be left with the necessary utensils inside the working chamber, the light turned on, and the lid partly open to replicate subsequent experimental settings. To achieve optimal sectioning, an array of different parameters were tested including different settings of the cutting angle, temperature settings from −20 to −40 °C, and a cutting thickness between 5 and 15 µm. In our hands, the optimal settings were those described in the Methods section. These produced an optimal balance between preserving tissue morphology and acquiring sufficient tissue for RNA harvest. We also found that gently warming the PEN-membrane slides by briefly placing an index finger on the well side of the slide

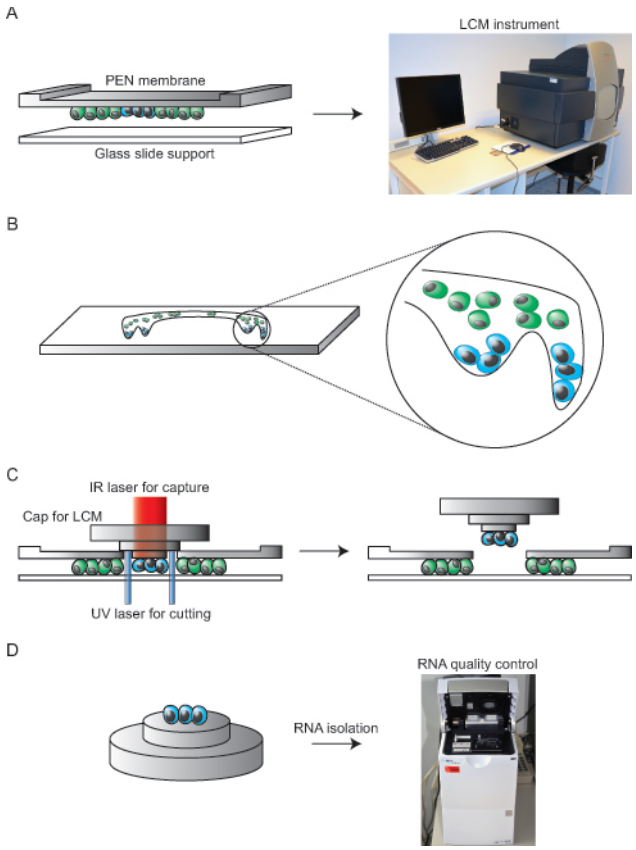

Figure 3. Laser capture microdissection of limbal crypt cells. **A**: Diagram depicting the tissue section sandwiched between the polyethylene naphtalate (PEN) membrane and a microscope glass slide. **B**: Before the capture, the basal limbal crypt cells (blue) and the corneal cells (green) are identified microscopically based on their histological structure. **C**: The basal crypt cells are fixed to the cap by applying infrared (IR) laser pulses (red), melting the cap focally to the PEN membrane, after which the cells are dissected from the remaining tissue by cutting around the area with an ultraviolet (UV) laser (blue bars). **D**: The cap with the cells is lifted from the remaining PEN membrane, the capture of the basal crypts cells is confirmed with microscopic inspection, and the cells are processed for RNA.

(opposite of the placement of the tissue section) facilitated the adherence of the cryosections to the slides.

As RNase activity depends on an aqueous environment and furthermore is inhibited by cold temperatures, the described protocols were optimized to use cold ethanol-based staining solutions. In the comparison between staining with either cresyl violet or with HistoGene, we found that the first method yielded higher-quality RNA than staining with HistoGene. In particular, the RIN numbers were on average 1.5 higher than those after HistoGene staining. These findings appear to be supported by previous observations [9].

After we finalized the staining with a drying step in xylene, we found that the slides should be maintained at room temperature. Recooling the cryosections led to condensation on the slides, and should be avoided since moisture promotes activation of endogenous RNases. Drying the tissue sections was also important for efficient capture by the IR laser. The outlined protocol for preparing histological sections for LCM is simple and fast, and, importantly, the morphological detail of stained cryosections permitted precise identification of all cellular compartments within the cornea.

Laser capture microdissection: LCM makes it possible to isolate and characterize specific cell populations within complex tissue surroundings. The cells below the applied IR laser reach peak temperatures of 90 °C for less than 200 ms, and this brief thermal peak does not adversely affect biomolecules for later use in downstream applications [5]. For the LCM procedure, we found that the Arcturus Veritas should be turned on at least 1 h before use. The LCM protocol involved a combination of UV cutting and IR capture. For optimal results, the IR laser capture should always precede UV cutting. The IR laser was focused cap-down at 10× magnification before capture. The UV laser was also focused before the experiments began; however, UV cutting should always be performed at 40× magnification for optimal focusing of

energy. It is also important to use the minimal necessary energy setting to avoid heat-induced damage of tissue adjacent to the cut. In our hands, using the control tissue scrapes, we observed that the LCM procedure decreased RIN values by about 1. Since this deterioration cannot be avoided, the input material for LCM should exhibit the highest possible RNA integrity.

In this protocol, the LCM procedure was performed on tissue sections sandwiched between a sterile PEN membrane and a sterile supporting glass slide. This arrangement provided a multitude of benefits. In particular, it protected the cells from airborne RNase contamination through dust particles and minimized the risk of harvesting unwanted cells. In addition, the flat surface of the PEN membrane allowed for the use of CapSure Macro Caps without guide rails, which furnished a larger capture area than with CapSure HS LCM Caps. Placing the cap directly on the flat PEN membrane also enhanced laser focusing on the thin corneal sections due to absence of cap tilting, and finally, the sandwich setup along with combined IR capture and UV cutting eliminated sheer and substratum forces, thus permitted absolute capture efficiency.

Using our protocol, we processed three sections in parallel within a time frame of 1 h and obtained around 1 ng of RNA. Since the lower limit for the current amplification methods is only 500 pg RNA, our approach appears sufficiently robust to provide enough RNA for reliable transcriptional profiling. If desired, however, the harvest can be scaled up by processing more sections. In our own hands, we obtained high-quality libraries from a pool of 15 cryosections [8].

Quality control of protocol: Using this protocol, RNA can be obtained with RIN numbers of up to 8.5. Importantly, RIN values above 7 are generally considered good for microarray analysis or deep sequencing. Furthermore, the procedures detailed in this paper appeared superior to previously published protocols in which RNA isolated from limbal cells harvested by LCM yielded RIN values of 5.1–7.5 [11,12].

When attempting to characterize LESCs in situ, numerous issues have to be addressed. First, as the expression profile of a tissue changes rapidly when it is exposed to cold ischemia, the material has to be processed as soon as possible after either the patient's death or after globe removal. With the combined use of the corneal slicer and flash-freezing within the operating theater, the time from removal to freezing is less than 5 min. Second, as the stem cells constitute only a small subpopulation of cells within the corneal tissue, measures have to be taken to ideally remove all non-stem cells. Using LCM, we removed the majority of the contaminating cells, and analyzed only the cells within the basal limbal crypts, thus significantly enhancing the proportion of stem cells within the analyzed cell population. The suitability of this protocol is underscored by RIN values of up to 8.5, indicating the relatively small degradation of RNA throughout the entire pipeline, including harvest, transport, storage, sectioning, histological staining, LCM, and RNA isolation. Although this protocol focuses on limbal cells, the procedure is equally suitable for analyzing any subpopulation of cells within the cornea.

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