Frozen, Pre-stripped Descemet Membrane Endothelial Keratoplasty (DMEK) Grafts For Surgical Training

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ABSTRACT

Purpose: To test whether pre-stripped, previously frozen DMEK grafts can be used as a substitute for freshly prepared tissue for surgical training.

Methods: Twenty pre-stripped DMEK tissues were prepared according to standard protocols. Tissues were frozen and stored in Optisol-GS at minus 80°C. Thawed tissues were examined by DMEK trained cornea surgeons to evaluate tissue handling during graft preparation and tissue behavior after transplantation into donor whole eyes. The scroll width of previously frozen grafts was measured using a calibration grid and compared to previous studies performed using fresh tissue. Eight additional tissues were utilized for cell viability staining and analysis of scrolling tendencies in grafts devoid of endothelium.

Results: Tissue donor age ranged from 52-86 years, with tissue frozen-to-analysis times ranging from 3-57 days. Trained DMEK surgeons noted no differences between tissue handling of previously frozen DMEK grafts compared to fresh tissue. Grafts were successfully trephinated, stained with trypan blue, and prepared similarly to fresh tissue. DMEK graft scroll widths ranged from 1.11 – 2.30 mm (average=1.70 ± 3.4 mm), and were similar to previous reports examining fresh tissues. After injection into donor eyes, grafts were able to be unfolded and positioned using standard DMEK surgical techniques.

Conclusion: Previously frozen, pre-stripped DMEK tissue handles similarly to freshly prepared tissue and can be used for surgeon training. The availability of frozen pre-stripped tissue will improve accessibility for surgeon training.

Key Words: DMEK; endothelial keratoplasty; pre-stripped DMEK grafts; tissue for surgeon training; DMEK scroll tightness; corneal transplant

Adoption of DMEK has been on the rise, but a major obstacle to increased utilization of the surgery is its steep learning curve. DMEK skills-transfer labs are an effective venue for surgeons to begin learning the procedure, but from the perspective of an eye bank's workflow, they can require significant resources because of the high volume of DMEK tissue typically required in a short interval of time. For example, a DMEK training course held annually at one of the national ophthalmic meetings can require an eye bank to acquire and prepare between 25 to 30 tissues within days of the event, in addition to the usual volume of tissues prepared for transplant every week.

Freezing DMEK tissue after pre-stripping for later use in surgeon training could decrease the burden on eye banks, as this tissue could be prepared, stored, and used at a moment's notice. However, it is unknown whether frozen DMEK tissue behaves comparably to fresh DMEK tissue in a skills-transfer setting.

The purpose of this study was to examine whether eye bank prepared DMEK grafts that were frozen and stored could be thawed and substituted for fresh DMEK tissue in a wet lab. Frozen DMEK tissues were thawed and then evaluated for scroll tightness, retention of elasticity, and to see whether an S-stamp could be visualized to confirm correct orientation during wet lab sessions.

MATERIALS AND METHODS

Donor Tissue Selection

Research consent was obtained for donor tissues used in the study, all of which were deemed unsuitable for transplant due to positive serology results. Tissues suitable for DMEK preparation were from donors age 50 and over with no penetrating scars within the central 8.0 mm graft zone. These criteria are the same that are used for DMEK transplant tissue. Endothelial cell density (ECD) requirements were waived for this study in order to capture a wide range of ECD values.

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Preparation of Frozen DMEK Grafts

DMEK tissue was prepared by experienced eye bank technicians, using a standardized forceps stripping technique and an S-stamp.[1,2] Post DMEK preparation, tissue was placed in a viewing chamber filled 90% with Optisol-GS (Bausch and Lomb, St. Louis, Mo. USA, USA). The viewing chambers containing the prepared tissues were then stored lid-side down, to ensure the cornea would be covered in Optisol-GS, in a minus 80°C freezer. Prior to use, frozen tissues were removed from the freezer and thawed at room temperature for 6 hours.

Preparation of Frozen DMEK Grafts Devoid of Endothelium

To determine if the endothelial junctions possibly contribute to DMEK graft scrolling, several donor corneas were prepared with the endothelium wiped off with an EYETEC® PVA sponge (Network Medical Products, North Stonington, CT, USA) prior to DMEK graft preparation. DMEK grafts without endothelium were then prepared as described above, and subjected to the same measurements and evaluation as described below, including vital dye staining and image acquisition.

Use of Frozen DMEK Tissue

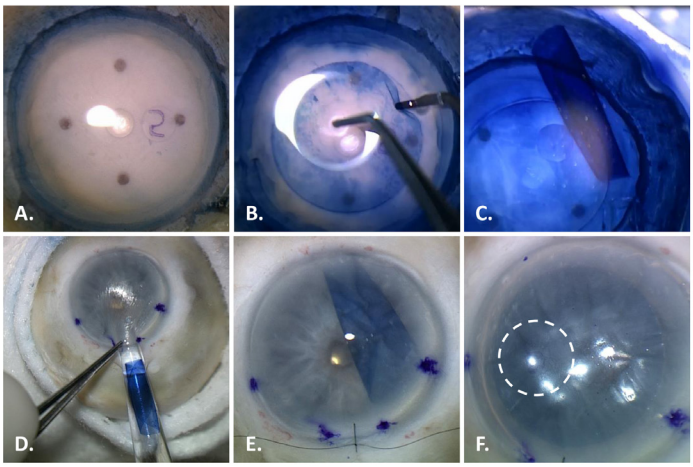
Wet labs using frozen DMEK tissues were performed by corneal surgeons trained in DMEK surgery (DLD and KD). Grafts were prepared using the same standardized technique utilized in DMEK surgery (see Fig. 1),[3] with a minor modification in that thawed tissues were punched using an 8.0 mm Barron Hessburg trephine (Barron Precision Instruments, Grand Blanc, MI, USA). Residual Optisol-GS was wicked away and trypan blue 0.06% solution (C-Blue, Stephens Instruments, Lexington, KY, USA) was used to stain the graft for visualization of the graft borders. The grafts were washed with Balanced Salt Solution (BSS, Alcon, Ft. Worth, TX, USA) and then punched to generate a circular graft from the central region of the cornea. Excess Descemet membrane outside of the graft zone was removed. The graft was peeled away from the underlying stroma and laid back down inside the corneal-sclera cap. BSS was removed and additional trypan blue solution was added to the cap and the immersed DMEK graft was allowed to scroll into its natural formation. Each graft was stained with immersion in trypan blue solution for four minutes. After four minutes, the trypan blue solution was carefully removed and BSS was added to the cap until the DMEK graft was visible as a free-floating scroll.

Scroll Thickness Measurements

For scroll tightness measurements, each graft was drawn into a Straiko Modified Jones tube (Gunther Weiss Scientific Glassblowing, Portland, OR, USA) and transferred into a petri dish filled with 50 mL of BSS. A slide with square grids spaced 2.0 mm apart was placed underneath the DMEK graft and petri dish. Images of the scrolls and grid were taken and scroll width was measured using FIJI[4](see Fig. 2). The 'Set Scale' function in FIJI was used to define the scale based on the spacing of the grid. Scroll width was taken at the widest point on the graft by two independent readers and averaged.

***Figure 1**: Surgical handling of previously frozen grafts.*

A previously frozen DMEK graft prepared and used for surgeon training in combination with a donor eye.



***A.** Thawed graft upon removal from viewing chamber.*

***B.** Graft being lifted away from the underlying stroma. The graft was stained with Trypan blue for 30 seconds after trephination to reveal the graft edge.*

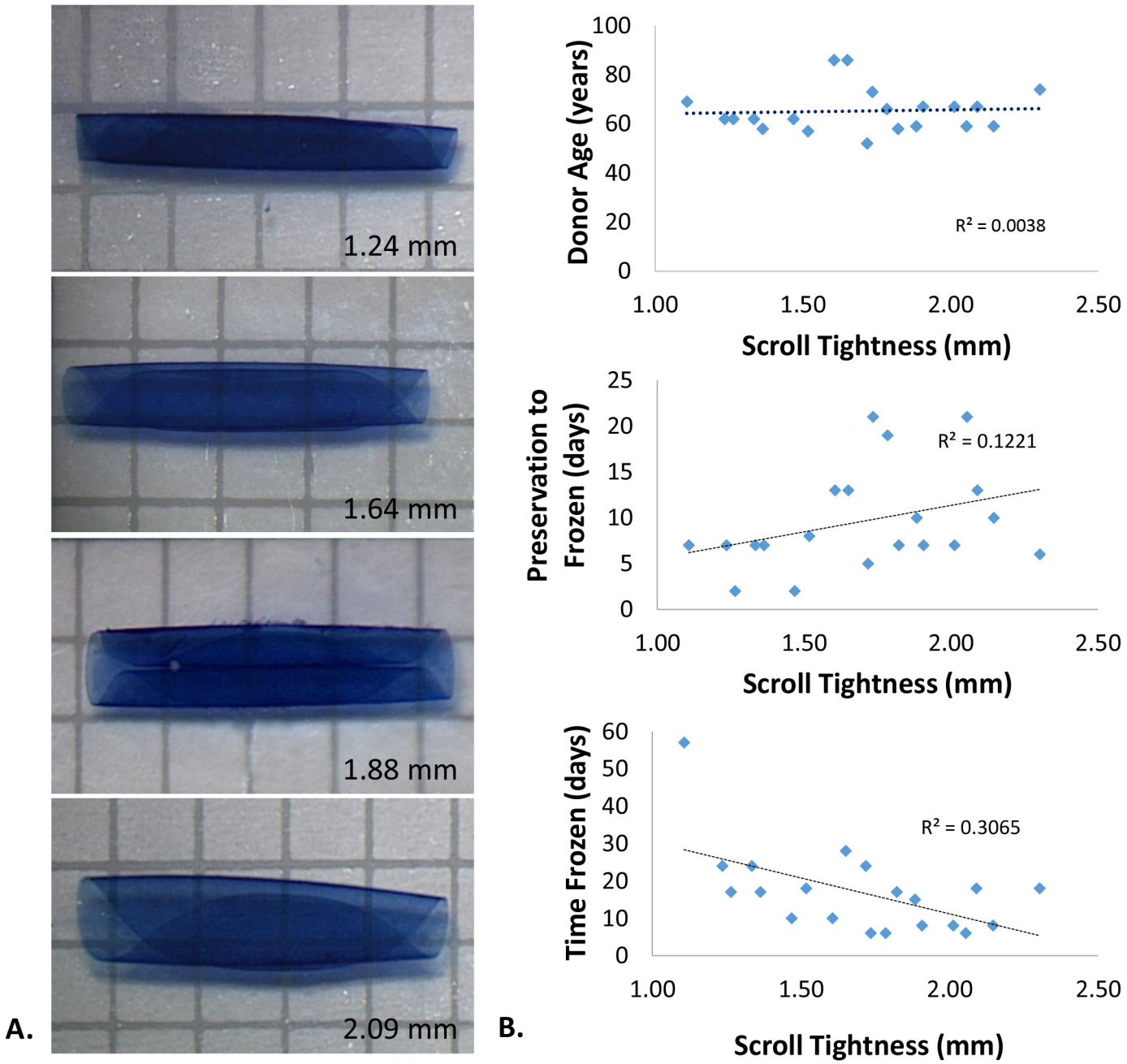
***C.** Graft in scrolled conformation after being stained with Trypan blue for 4 minutes and washed with BSS.*

***D.** Delivery of graft into a donor eye using a Modified Straiko injector.*

***E.** Graft inside the anterior chamber during unfolding.*

***F.** Graft fully unfolded and attached to the stroma after injection of an air bubble. The S-stamp (white outline) can be seen and indicates that the graft is in the proper orientation.*

***Figure 2**: Scroll width measurements*



***A.** DMEK scrolls floating inside a BSS-filled dish set on top of a measurement grid. The grid lines are 2.0 mm apart. The numbers on the bottom right corner of each panel indicate the scroll width as measured at the widest point. (Top to Bottom) An example of tight scroll in this series (62 year old donor), a tri-fold scroll (86 year old donor), a double scroll (59 year old donor), a loose scroll (67 year old donor).*

***B.** Correlation between donor tissue parameters and scroll width.*

Surgical Handling Simulations

A cadaveric whole eye was prepared with epithelial scraping and the application of glycerin drops to the cornea for improved visualization. DMEK grafts were drawn into the Modified Straiko Jones tube for delivery into the anterior chamber to examine tissue behavior while using various techniques (See Video, Supplemental Digital Content 1, for an illustration of these techniques). These include, but are not limited, to:

- Quick, staccato taps on the cornea, which generate fluid waves that can unfurl the tissue.5

- Dirisamer maneuver6: one cannula shallows the chamber in an area where the tissue is already unfurled, effectively isolating (but not compressing) the tissue down, while staccato taps are performed with the other cannula to unscroll tissue that is now in a deeper part of the chamber.
- Compress and release: done by compressing the cornea peripheral to a scrolled edge and then quickly releasing the cannula in the direction where tissue unscrolling movement is desired.
- Wound flicking: fluid currents can be created if a cannula is inserted into an incision, and used to flick the wound open while the cannula is being removed. This creates an exit current that will bring the tissue closer to the wound being flicked.
- Rolling over upside down tissue: involves deepening the anterior chamber and using gentle bursts of BSS along either the corneal or iris plane to flip the tissue over into the correct orientation.
- Golf strokes: quick strokes at the limbus with a cannula to generate a fluid wave that will move the tissue forward, away from the limbus.
- Tissue "walking": two cannulas are used to move the tissue, by using small, gentle strokes with alternating hands to shuffle the graft forward.

These DMEK techniques are also well demonstrated in prior open-access videos.[7]

Vital Dye Staining and Image Acquisition

Images of the trypan blue stained grafts were captured using a Zeiss OPMIMD S-5 microscope (Zeiss, Thornwood, NY. USA) equipped with an Optronics Microcast HD digital camera (Optronics, Goleta, CA. USA). To assess cell viability, the same grafts were stained with Calcein-AM (2.5 mg/mL; ThermoFisher, Grand Island, NY, USA) for 40 minutes at room-temperature followed by gentle rinsing with BSS. Grafts were stained for an additional 15 minutes with Propidium Iodide (PI) (50 mg/mL; ThermoFisher, Grand Island, NY, USA), which stains dead cells. Grafts were punched with an 8.0 mm trephine and then gently transferred onto a glass slide containing a bed of viscoelastic (Occulon, Stephens Instruments, Lexington, KY, USA). Fluorescent images were captured using a XDY-1 inverted fluorescent microscope (Alltion, Wuzhou, China) and 50-70 images were acquired for each graft at 40X magnification. Images were combined using Adobe Photoshop Elements 14.0 (Adobe Systems, San Jose, CA, USA).

Statistical Analysis

All statistical analysis was performed using SPSS version 23 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2013 (Microsoft Corp, Redmond, WA, USA). Pearson's correlations coefficients were derived to evaluate scroll tightness with tissue characteristics. All statistics were two sided and alpha was set to 0.05.

RESULTS

Donor Tissue Demographics

Twenty-eight "frozen" grafts were evaluated in this study (20 for scroll tightness, 4 for cell viability staining, and 4 for the experiments where the endothelium was purposely removed). Donor age for tissues used in this study ranged from 52-86 years (average=66 years). Death-to-preservation times for tissues were between 5.5 and 26 hours (average=14.2 hours). Preservation-to-frozen times ranged from 2-21 days (average=9 days), and frozen-to-analysis times ranged from 3-57 days (average=17 days). ECD for tissues examined prior to freezing ranged from 1350 - 2941 cells/mm^2, with an average of 2318 cells/mm^2 for all tissues examined.

Graft Handling in Donor Whole Eyes

The S-stamp was visible on all previously frozen tissues examined. There were no torn grafts during final tissue preparation prior to loading the grafts into the injector. Maneuvers employed in surgery to unscroll and center the tissue were successfully completed with all tissues.

Tissue Scrolling and Scroll Tightness

The average scroll tightness for all 20 grafts examined in this series was 1.70 ± 0.34 mm (range=1.11-2.30 mm, Fig. 2, Table 1). All grafts were punched with an 8.0 mm trephine, resulting in an average scroll length of 8.85 ± 0.30 mm (range=8.58 – 9.53 mm). We did not find a significant correlation between donor age and scroll tightness (Fig. 2B, R2=0.004, p=<0.001). We observed a weak, but non-significant correlation between preservation-to-frozen times and scroll tightness. (Fig. 2B, R2=0.12, p=0.13), and a moderate and significant correlation between frozen-to-use times and scroll tightness (Fig. 2B, R2=0.36, p=0.01).

Endothelium of Frozen Pre-stripped Tissues

Propidium Iodide staining of 4 previously frozen, pre-stripped DMEK grafts revealed large areas of cell death

***Table 1**. Donor Characteristic and Scroll Tightness*

Tissue Number	Average Width (mm)	Donor Age	Preservation to Frozen (Days)	Frozen to Use (Days)	Endothelial Cell Density
1	1.11	69	7	57	-
2	1.24	62	7	24	2611
3	1.26	62	2	17	2755
4	1.33	62	7	24	2538
5	1.36	58	7	17	-
6	1.47	62	2	10	2857
7	1.52	57	8	18	2128
8	1.61	86	13	10	-
9	1.65	86	13	28	2494
10	1.72	52	5	24	2141
11	1.73	73	21	6	1984
12	1.78	66	19	6	2639
13	1.82	58	7	17	-
14	1.88	59	10	15	-
15	1.91	67	7	8	1953
16	2.01	67	7	8	1350
17	2.05	59	21	6	2096
18	2.09	67	13	18	-
19	2.15	59	10	8	-
20	2.30	74	6	18	2584
Average:	1.70 ± 0.34	65.25 ± 8.97	9.6 ± 5.5	16.9 ± 11.6	2317 ± 420
Range:	1.11-2.30	52-86	2-21	6-57	1350-2857

as well as areas devoid of cells (Fig. 3A-B). No Calcein-AM staining was detected, and areas devoid of cells appeared as dark areas with no Calcein-AM and no PI staining (Fig. 3B).

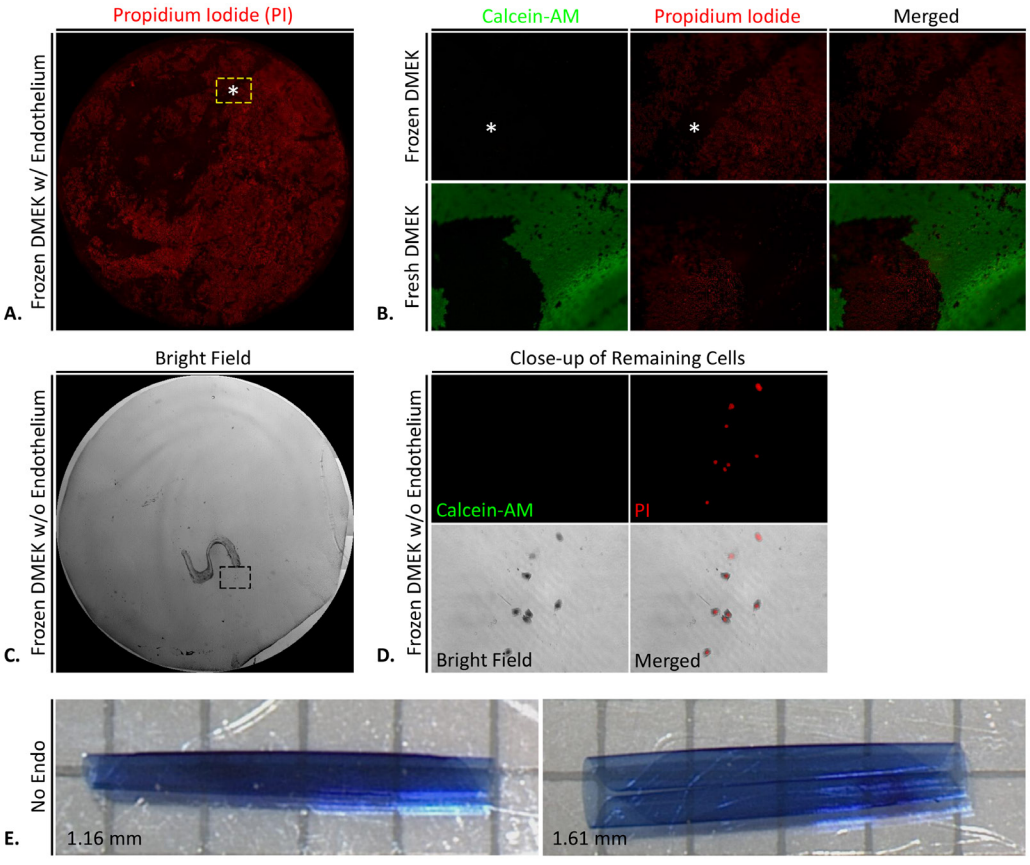
For the 4 tissues where endothelial cells were intentionally removed prior to processing, light microscopy revealed grafts with bare Descemet membrane. No Calcein-AM signal was detectable on the grafts, and a few remaining cells showed positive PI staining (Fig. 3C-D). The average scroll tightness for the four grafts devoid of endothelium was 1.43 ± 0.34 mm (range=1.16-1.61 mm, donor age range=70 – 75 years), and the average length was 8.70 ± 0.12 mm (range=8.54-8.82 mm) (Fig. 3E).

DISCUSSION

A surgical wet lab is an essential tool to help surgeons learn and practice new techniques

***Figure 3**: Vital dye staining of a previously frozen grafts.*

Previously frozen graft stained for Trypan blue, Calcein-AM, and Propidium Iodide (PI).



***A.** PI staining of the 8.0 mm punched graft. The region outlined in yellow is enlarged in panel B.*

***B.** (Top) Calcein-AM and PI staining of a previously frozen graft. The dark region marked with an * is an area that contain no cells (i.e. bare Descement membrane). (Bottom) An example of vital dye staining of live tissue. An area containing a lot of cell death was chosen to demonstrate the difference in staining pattern of Calcein-AM and PI.*

***C.** Bright Field image of a graft where the endothelium was removed prior to DMEK preparation and frozen storage. The region outlined in black is enlarged in panel D.*

***D.** Close-up of a small area with a few remaining endothelial cells*

***E.** Examples of DMEK scrolls from tissues where the endothelium was wiped prior to preparation and frozen storage. The numbers on the bottom left corner of each panel indicate the scroll width at the widest point.*

prior to implementing them with patients. Many new methods and technologies have been developed to increase efficiency and ease of training in this setting. Using frozen tissue, as we have proposed, is another important development that will aid in surgeon training. DMEK wet labs currently use fresh, unfrozen tissue for physician utilization. The results of our study show that frozen DMEK tissue, when thawed, behaves like fresh tissue and can also be used in this arena.

An increasing number of DMEK surgeons in the United States are utilizing pre-stripped DMEK tissue for surgery,[8] which still requires final steps of graft preparation in the operating room. These steps include tissue trephination, removal of excess peripheral Descemet membrane, peeling of the graft from the underlying stroma, staining the tissue, and drawing the tissue up into an injector. All 20 frozen grafts in this series, when thawed, behaved exactly like fresh tissue during these steps (Fig. 1, See Video, Supplemental Digital Content 1, for a demonstration of these steps). Thus, previously frozen, pre-stripped DMEK grafts seems to be a suitable alternative to fresh tissue for final graft preparation training.

After thawing, all 20 tissues scrolled in the same predictable way that fresh tissue scrolls, with a broad range of scroll tightness (Fig. 2). These scrolls, once injected into the eye, exhibited the same unscrolling tendencies as fresh tissue and responded similarly to no-touch graft unfolding and manipulation techniques (See Video, Supplemental Digital Content 1, for a presentation of these techniques).

Previous reports have suggested that the scroll tightness of DMEK grafts may be influenced by tissue donor age and endothelial cell density.[9,10] However, no published studies have examined whether endothelial cell viability or the presence of cells is required for tissue scrolling. To this end, our results suggest that the tendency of a DMEK graft to scroll does not seem to be greatly influenced, if at all, by viable endothelium, as freezing tissue clearly kills these cells (Fig. 3A-B).

Although the frozen DMEK grafts had no viable endothelial cells, they were not completely devoid of cells. We questioned at this point whether any endothelium, viable or not, was necessary for scrolling. 4 DMEK grafts without endothelium were then prepared and evaluated as described above, including vital dye staining and image acquisition. Analysis showed similar tissue scrolling and unfurling tendencies when compared to tissue with endothelium present. This demonstrates, with a limited sample size, that the endothelium does not even need to be present for a tissue to scroll. Thus, the tendency of a tissue to scroll is dependent on factors such as donor age and Descemet membrane thickness[9,10] rather than the presence of cells, viable or not.

Lions VisionGift has already provided 46 frozen tissues for 2 wet labs in 2016, one at the Massachusetts Eye and Ear Infirmary and the other at the annual meeting of the American Academy of Ophthalmology in Chicago. Both of these training events were successful with no problems attributed to using frozen tissue. The ability to use frozen tissue will be extremely beneficial to eye banks, as it will relieve demand for large quantities of fresh tissue days before an event. With frozen DMEK tissue, an eye bank can accumulate and store tissue designated for education for weeks, or months, prior to an event. In addition, tissue that would otherwise be discarded may be pre-stripped, frozen, and stored for later use for DMEK surgical training. The daily work demand of an eye bank technician can be quite variable, with some days being extremely busy and others with a more modest workload. Freezing DMEK tissue will help to distribute that workload more evenly.

A limitation to our study is that we only used tissues frozen for a maximum of approximately 2 months. We do not know how tissue will behave beyond this storage time point. It is possible that a prolonged storage time may induce changes in DMEK tissues stored in the freezer and make them unusable for training purposes. Further, while we examined correlations between various tissue parameters and DMEK scroll tightness, we must emphasize that the results are based on a limited sample size in a narrow age range (i.e. DMEK suitable range of 52 – 86 years). We've also shown that the tendency for DMEK grafts to scroll is independent of endothelial cell viability and the presence of cell bodies. However, we have not directly addressed these parameters with large numbers of research tissues. Such studies would be interesting, but beyond the scope of this manuscript.

In conclusion, previously frozen pre-stripped tissue behaves like fresh tissue and may be used for DMEK wet

labs to practice every step of the procedure. The availability of frozen tissue will increase ease of training and may help eye banks alleviate non-transplant workload when viable endothelial cells are not necessary.

Supplemental Digital Content 1. Video that demonstrates frozen DMEK tissue being utilized to practice DMEK graft preparation, insertion, and manipulation using various techniques.

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