

1 **A COMPARATIVE STUDY ON DIFFERENT DMEK GRAFT PREPARATION TECHNIQUES**

2

3 **Authors**

4 Mohit Parekh<sup>1</sup>, Davide Borroni<sup>2,3</sup>, Alessandro Ruzza<sup>1</sup>, Hannah J Levis<sup>4</sup>, Stefano Ferrari<sup>1</sup>,  
5 Diego Ponzin<sup>1</sup> and Vito Romano<sup>5</sup>

6

7

8 **Affiliations**

9 <sup>1</sup>International Center for Ocular Physiopathology, The Veneto Eye Bank Foundation,  
10 Venice, Italy

11 <sup>2</sup>Department of Ophthalmology, Riga Stradins University, Riga, Latvia

12 <sup>3</sup>Department of Genetics, Riga Stradins University, Riga, Latvia

13 <sup>4</sup>Department of Eye and Vision Science, Institute of Ageing and Chronic Disease,  
14 University of Liverpool, Liverpool, UK

15 <sup>5</sup>Corneal and External Eye Service, St. Paul's Eye Unit, Royal Liverpool University  
16 Hospital, Liverpool, UK

17

18

19 **Correspondence**

20 Mohit Parekh, PhD

21 International Centre for Ocular Physiopathology

22 Fondazione Banca degli Occhi del Veneto, Onlus

23 Via Paccagnella 11, Padiglione Rama

24 30174, Zelarino, Venice, Italy

25 Tel: +39 0419656473

26 Fax: +39 0419656471

27 e-mail: mohit.parekh@fbov.it

28

29

30 **Running title**

31 DMEK graft preparation comparison

32

33

34 **Word count**

35 3435

1 **ABSTRACT**

2

3 **Purpose:** To compare different Descemet Membrane Endothelial Keratoplasty  
4 (DMEK) graft preparation methods.

5 **Methods:** *Stripping from the trabecular meshwork (M1)* using epithelial spatula;  
6 *stripping by scoring the peripheral endothelium (M2)* using Sinsky hook; *stripping by*  
7 *punch method (M3)* using donor punch; *Submerged Hydro-Separation (M4)*; and  
8 *pneumatic dissection method (M5)* were evaluated. Preparation time, costs,  
9 endothelial cell loss (ECL) post-preparation, cell death and morphology were  
10 compared. Hoechst/Ethidium/Calcein AM (HEC) staining and Zonula Occludens-1 (ZO-  
11 1) expression was analyzed. Statistical analysis was performed using Anova test and;  
12 Tukey as post-hoc test.

13 **Results:** 35 corneas (7 per group) were used. ECL represented as Mean(SD), in M1,  
14 M2, M3, M4 and M5 was 2.7(5.0), 3.0(7.4), 1.2(7.4), 3.3(7.3) and 4.1(7.1)%  
15 respectively not showing any difference between the groups ( $p=0.96$ ). A significantly  
16 higher cell death ( $p<0.05$ ) was observed in M4 and M5 compared to M1, M2 and M3.  
17 Graft preparation time was significantly shorter in M4 and M5 and longest in M3  
18 ( $p<0.05$ ). M3 was the most expensive preparation technique. Minimum pleomorphic  
19 cells were observed in M1, M2 and M3 whereas moderate pleomorphism was seen in  
20 M4 and M5. HEC staining showed high Ethidium positivity (dead cells) in M4 and M5  
21 with minimum positivity in M1, M2 and M3. ZO-1 was expressed in all the conditions  
22 except the denuded areas.

23 **Conclusion:** Graft preparation using *Sinsky hook (M2)* and *donor punch (M3)* are  
24 reliable methods in terms of efficiency and quality with acceptable range of ECL. The  
25 preparation time and associated costs could be a limitation for M3.

26

27 **KEYWORDS**

28 DMEK; graft preparation; comparison

29

1 **INTRODUCTION**

2 Descemet Membrane Endothelial Keratoplasty (DMEK) has gained popularity for  
3 many surgeons to treat specific endothelial failure cases. DMEK offers early  
4 rehabilitation with optimal visual acuity [Melles et al. 2006; Kruse et al. 2011; Price et  
5 al. 2013]. Its minimally invasive nature and low immunologic rejection rate make this  
6 technique favourable for surgeons (Anshu et al. 2012). Even with these advantages,  
7 the technique still requires refinement in terms of graft preparation, delivery,  
8 unfolding, and positioning in the recipient eye (Terry 2012; Gorovoy 2014). Recently,  
9 the eye banks have been requested with pre-stripped DMEK tissues (Deng et al. 2015;  
10 Kobayashi et al. 2015) that aim to reduce the surgical efforts, time, and costs in the  
11 theatre. With such requests, the eye banks are challenged to find a standardized  
12 method in terms of feasibility of preparation, good graft quality, availability of the  
13 instruments and associated costs. Earlier, air bubble (Busin et al. 2010) and liquid  
14 bubble (Parekh et al. 2014) techniques to separate the stroma and Descemet  
15 Membrane (DM) has been described. Dapena et al. (Dapena et al. 2011) and  
16 Groeneveld et al. (Groeneveld et al. 2013) also suggested no-touch method for DMEK  
17 stripping. It was noted from the literature that the bubble and stripping techniques  
18 are uptaken easily and have been used for DMEK graft preparation. The following  
19 study therefore describes five methods that are slight modifications of the  
20 conventional bubble and stripping techniques and determine the effects of each  
21 technique on endothelial morphology and cell loss with an intention to highlight the  
22 most feasible option in terms of costs, quality and efficacy allowing eye bankers and  
23 surgeons to optimize the best choice for DMEK graft preparation.

24  
25

1 **MATERIALS AND METHODS**

2 *Ethical Statement*

3 Human donor corneas [n=35] were collected from the Veneto Eye Bank Foundation,  
4 Mestre, Italy (FBOV) with written consent from the donors' next-of-kin for use in  
5 research. The study followed the tenets of 2013 Declaration of Helsinki.

6

7 *Donor characteristics and tissue preservation*

8 Donor characteristics were obtained from the FBOV database. All the corneas were  
9 preserved at 31°C in tissue culture medium (TCM) that was composed of 2% newborn  
10 calf serum with MEM-Earle as a base medium along with 25 mM HEPES Buffer, 26 mM  
11 sodium bicarbonate, 1 mM pyruvate, 2 mM glutamine, 250 ng/mL amphotericin B,  
12 100 IU/mL penicillin G, and 100 mg/mL streptomycin. TCM was prepared at FBOV with  
13 full regulatory compliance.

14

15 *Endothelial evaluation*

16 The endothelium of all the samples was stained using trypan blue (0.25%) for 20  
17 seconds and washed with phosphate buffered saline (PBS). The corneas were placed  
18 in a sterile petri dish containing a hypotonic sucrose solution (to increase definition of  
19 cell borders) with the epithelium uppermost. To estimate endothelial cell density  
20 (ECD), the cells in five 1-mm<sup>2</sup> squares of a 10 X 10 mm reticule inserted in the eyepiece  
21 of an inverted microscope (Primovert; Zeiss, Jena, Germany) were counted manually  
22 at 100X magnification. The number of blue-stained (trypan blue positive cells - TBPCs)  
23 cells allowed percentage cell death to be determined. The ECD and cell death were  
24 counted both before and after DM stripping.

25

26 *Alizarin red staining [n=3 per method]*

27 After DMEK preparation, the endothelium was stained (immersed) with alizarin red S  
28 (Sigma Aldrich – A3757, Missouri, United States) for approximately 5 minutes, washed  
29 with PBS, and viewed at 100x magnification using an inverted microscope to observe  
30 the morphology (pleomorphism, polymegethism, and hexagonality) of the cells.

31

32 *Hoechst, Ethidium homodimer and Calcein AM (HEC) staining to determine live/dead  
33 cells [n=2 per method]*

34 The tissues after trypan blue staining were washed with PBS prior to HEC staining. 4  
35 µL of Hoechst 33342 (H) (Thermo Fisher Scientific, Rochester, NY, USA), 4 µL of  
36 Ethidium Homodimer EthD-1 (E) and 2 µL of Calcein AM (C) (Live/Dead  
37 viability/cytotoxicity kit, Thermo Fisher Scientific, Rochester, NY, USA) was mixed in 1  
38 mL of PBS and mixed well. 300 µL of the final solution was directly added on the  
39 endothelium of the DMEK tissue resting on the cornea and incubated at room  
40 temperature in the dark for 30 minutes. The DMEK lenticule was excised and placed  
41 on a slide with the endothelium uppermost. Four radial cuts were made to obtain a

1 flat mount of the tissue on the slide and covered with coverslips without the mounting  
2 medium. The endothelium was viewed at 100x magnification of Nikon Eclipse Ti-E  
3 (Nikon, Burgerweeshuispad, Amsterdam) using NIS Elements software (Nikon).

4  
5 *Expression of tight junctions using Zonula Occludens-1 (ZO-1) marker [n=2 per method]*

6 The tissues were washed with PBS and fixed in 4% paraformaldehyde (PFA) at room  
7 temperature (RT) for 10 minutes. The cells were permeabilized with 0.1% Triton X-100  
8 in PBS for 10 minutes. After blocking with 1% bovine serum albumin for 1 hour at RT,  
9 the tissues were incubated with 0.1% bovine serum albumin and primary antibody  
10 [anti-ZO-1, 1:200 (ZO1-1A12 Alexa Fluor 488 conjugate; Invitrogen, Carlsbad,  
11 California, USA)] for 3 hours at room temperature. After each step, the cells were  
12 washed thrice with 1X PBS. The tissue was flat mounted on the slide and fixed with a  
13 mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) to stain the  
14 nuclei. Cells were examined under Nikon Eclipse Ti-E (Nikon, Burgerweeshuispad,  
15 Amsterdam) microscope at 100X magnification and the images were captured with  
16 NIS Elements software (Nikon).

17  
18 The study was divided into the following five groups, each with 7 corneas. M1 was  
19 performed by DB, M2 by VR, M3 by AR, M4 and M5 by MP. Each technician has  
20 prepared more than 50 tissues with the method they were assigned and therefore  
21 their level of experience was deemed suitable for this study.

22  
23 *Stripping from the trabecular meshwork – Method 1*

24 A cornea was centered on a punch base using suction; however, it is not mandatory  
25 to use a suction base for this method. The tissue was stained with trypan blue for ease  
26 of visualization for 1 minute. The point of initiation (PoI) (starting point where the  
27 instrument is touched for incision) was 1mm away from the Trabecular Meshwork  
28 (TM) on the sclera. An epithelial spatula (Blink Medical Ltd., Birmingham, UK) was used  
29 to swipe the TM towards the cornea detaching the peripheral endothelium as  
30 observed in Figure 1A and 1B. The TM was excised completely exposing the periphery  
31 of the DMEK graft (Figure 1C). The DMEK was grasped at its periphery using a 120 mm  
32 straight pointed acute forceps (e.Janach, Como, Italy) and peeled using different  
33 peeling sites to release the DMEK tissue avoiding peripheral tears (Figure 1D). Note  
34 that trypan blue stain remains on the tissue keeping the tissue moist and there is no  
35 liquid (TCM) on the endothelium. The preparation method is shown in supplementary  
36 video 1.

37  
38 *Stripping by scoring the peripheral endothelium – Method 2*

39 A cornea was centered on a punch base using suction; however, it is not necessary to  
40 use the suction base in this method. The PoI of this technique is at the periphery of  
41 the DMEK graft, just before the TM as seen in Figures 1E and 1F. An Intra Ocular Lens

1 (IOL) manipulator [Sinsky] hook with blunt tip (Beaver-Visitec International Ltd.,  
2 Warwickshire, UK) was used to score the peripheral circumference on the endothelial  
3 side of the cornea (Figure 1G) detaching the periphery of the DMEK for further peeling.  
4 120mm straight pointed acute forceps (e.Janach, Como, Italy) was used to peel the  
5 DMEK graft using a single peel (superior to inferior) method (Figure 1H). The cells were  
6 kept moist with a single drop of PBS and were not totally submerged in the liquid. In  
7 challenging cases where the tissue was tightly attached to the stroma, multiple  
8 quadrant method (where the edge of the DMEK tissue is grasped at different sites and  
9 peeled in parts obtaining a single graft) was used. Note that unlike method 1, the  
10 tissue can be stained to see the scored area if necessary. The method is shown in  
11 supplementary video 2.

12

### 13 *Stripping by punch method – Method 3*

14 A cornea was centered on a punch base using suction. Vacuum was created using a  
15 syringe and the cornea was secured on the base. The PoI of this technique can vary  
16 depending on the donor punch (8.25-10.00 mm diameter) that is selected. For this  
17 study, we used our standard protocol with a 9.5 mm donor trephine making it the PoI  
18 (Figure 1I). The endothelium was gently tapped with the trephine (Moria, Antony,  
19 France) to make a superficial cut (Figure 1J). Trypan blue was applied on the  
20 endothelium for about 20 seconds to determine the trephined zone. Excess peripheral  
21 endothelium was removed using 120mm straight pointed acute forceps (e.Janach,  
22 Como, Italy) and the tissue re-stained to help see the non-endothelial part. The  
23 endothelium was kept moist during the entire procedure using TCM to create a film  
24 of fluid on the top of the endothelium. The membrane was slightly lifted at the  
25 periphery using a cleavage hook and the procedure was continued along the entire  
26 circumference of the trephined area to ensure limited peripheral tearing while peeling  
27 (Figure 1K). The DMEK tissue was stripped with a longitudinal movement using a 3-  
28 quadrant method, ensuring no stress lines were generated, thus reducing cell death.  
29 Once the DM was stripped, leaving a peripheral hinge, it was laid back onto the stroma  
30 (Figure 1L) as previously described (Parekh et al. 2016). The method is shown in  
31 supplementary video 3.

32

33 For Methods 1, 2 and 3, the DM was not completely stripped but left attached by a  
34 peripheral hinge. The DM was laid back onto the stroma for morphological and  
35 molecular analysis as described above.

36

### 37 *Submerged Hydro-Separation (SubHyS) Method – Method 4*

38 In this technique, a cornea was submerged in a sterile petri dish half-filled with the  
39 TCM to keep the endothelium moist throughout the procedure. The tissue was held  
40 at the sclera with sterile forceps. A needle (25 gauge) connected to a 1 mL syringe was  
41 inserted beneath the TM (PoI) (Figure 1M and 1N) and advanced radially in the

1 posterior stroma or stroma–Descemet membrane interface until the bevel of the  
2 needle was completely inserted. TCM from the syringe was injected into the stroma  
3 or the DM-stroma interface with pressure enough to separate the DM and the stroma.  
4 A small, clear bubble was visible (Figure 1O) with the initiation of the process. The  
5 bubble was enlarged with an increased pressure to achieve an approximately 10-11  
6 mm diameter bubble (Figure 1P). The liquid was removed by sucking the liquid back  
7 in the syringe (Parekh et al. 2014) and the tissue was used for further analysis. The  
8 method is shown in supplementary video 4.

9

#### 10 *Pneumatic dissection method – Method 5*

11 In this technique, a cornea was placed in a sterile petri dish with endothelial side  
12 upwards. Using a 25G needle attached to a 1 mL syringe, the needle was advanced in  
13 a tangential direction from the limbus (Figure 1Q) at the stroma-DM interface up to 2  
14 mm (Figure 1R). Air was injected to obtain a detachment of DM and stroma (Figure  
15 1S) and the bubble was enlarged up to the corneal periphery (Figure 1T). If the bubble  
16 was not achieved then another Pol was considered and the bubble was enlarged from  
17 that site until a complete bubble was obtained. The air was removed by sucking it back  
18 in the syringe and the tissue was used for further analysis (Busin et al. 2010). The  
19 method is shown in supplementary video 5.

20

#### 21 *Time and cost analysis*

22 The time for the preparation of graft was monitored by stopwatch and the costs were  
23 analyzed based on the most expensive instruments used in the technique.

24

#### 25 *Statistical analysis*

26 Comparisons between these groups were performed by one-way ANOVA followed by  
27 Tukey test as a post hoc analysis test. Data were deemed significantly different when  
28  $p < 0.05$ .

29

1 **RESULTS**

2 *Donor characteristics*

3 Average age of the donors, gender, post mortem hours and endothelial cell density  
4 (ECD) from each group is listed in table 1. All the tissues were preserved in TCM not  
5 more than 28 days at 31°C before the experiments.

6

7 *Preparation time*

8 The time required to prepare the grafts in all the groups was statistically significant  
9 ( $p < 0.05$ ) (Table 2). It was observed that M3 required longer graft preparation time  
10 compared to any other methods; M4 and M5 required the least time (Figure 2, Table  
11 2). M1 and M2 required almost same time to prepare the graft.

12

13 *Tissue wastage*

14 There was no total tissue wastage from M1, M2 and M3. Two tissues were partially  
15 peeled (60%) from M1 (although not lost completely) due to tight attachment of the  
16 DM to stroma compared to others. M2 and M3 did not show any tissue wastage. One  
17 tissue was lost due to over-filling of stroma with liquid and not forming a liquid bubble  
18 in M4. Two tissues were lost due to stromal swelling caused by air, which did not  
19 generate a bubble in M5. There were three sites used for pneumatic dissection to  
20 generate air bubbles but all three failed in M5. The wasted tissues were not  
21 considered in this study and other tissues were obtained for evaluation purposes using  
22 the same technique.

23

24 *Endothelial cell density, cell loss, cell death and morphology*

25 Minimal cell death (assessed by trypan blue, Fig 3) was observed in M1 (Figure 3A),  
26 M2 (Figure 3B) and M3 (Figure 3C). Both M4 (Figure 3D) and M5 (Figure 3E) resulted  
27 in higher cell death than the other methods with M5 showing the highest percentage  
28 of dead cells (Figure 4, table 2). Cell death was statistically significant ( $p < 0.05$ )  
29 between the groups (table 2). Endothelial cell loss was found to be highest in M5,  
30 however there was no significant difference between the groups (table 2).

31 The morphology in all the conditions did not differ pre- and post-preparation;  
32 however, there were some pleomorphic cells observed after Alizarin red staining  
33 (Figure 5A-5E). Denuded areas with no endothelial cells were noted after Alizarin red  
34 staining especially in M4 (Figure 5D) and M5 (Figure 5E).

35

36 *Cost*

37 Bubbling techniques (M4 and M5) were found to be least expensive with an  
38 approximate cost of \$. <1.0 (majorly being the syringe) compared with the stripping  
39 methods that used an epithelial spatula (\$. 8.0) or Sinsky hook (\$. 10.0). The most  
40 expensive technique was M3 that used donor punches costing up to \$. 150 also  
41 mentioned in table 3.

1 *Hoechst, Ethidium Homodimer and Calcein-AM staining*

2 Triple endothelial cell labeling with Hoechst 33342 (H), Ethidium Homodimer (E), and  
3 Calcein-AM (C) showed presence of 'E' in red representing the dead cells, blue  
4 representing the nuclei 'H' and green indicating the viable cells 'C'. Higher numbers of  
5 dead cells were observed in M4 (Figure 6D) and M5 (Figure 6E) [correlating with the  
6 data determined by trypan blue staining] compared with M1 (Figure 6A), M2 (Figure  
7 6B) and M3 (Figure 6C). The number of apoptotic cells was very low and as the cells  
8 were compact it was difficult to evaluate the exact number of apoptotic cells in  
9 different conditions.

10

11 *ZO-1 staining*

12 Expression of ZO-1 was observed in all the conditions M1-M5 (Figure 7A-7E  
13 respectively) and the hexagonality was partially maintained in all the cases wherever  
14 the cells were present. The function of tight junctions was hence checked and the  
15 morphology was correlated with Alizarin red staining. There were small, denuded  
16 areas due to the detachment of endothelial cells where ZO-1 was not expressed.

17

18

1 **DISCUSSION**

2 DMEK has shown advantages such as early visual rehabilitation rates and outcomes.  
3 Endothelial keratoplasty using pre-cut for DSAEK or pre-stripped for DMEK grafts have  
4 shown increased interest recently and the eye banks are increasingly requested to  
5 produce these challenging grafts (Ruzza et al. 2015; Parekh et al. 2017a). The most  
6 suitable method for DMEK graft preparation has become an area for debate. Although  
7 protocols to standardize graft excision and transplantation have been introduced  
8 earlier (Dapena et al. 2011 and Groeneveld et al. 2013) however, as there are many  
9 procedures followed for DMEK, an affordable and easy to prepare a good quality graft  
10 must be identified, as this is one of the reasons why uptake of DMEK by surgeons is  
11 growing at a slower rate. Eye bank technicians have experience preparing a high  
12 volume of corneas for selective transplantation on a daily basis and therefore a pre-  
13 prepared graft from the eye bank could be a valuable option for the surgeons to  
14 reduce the graft preparation time, tissue wastage and overall costs in the surgery  
15 (Ruzza et al. 2015). Using pre-prepared tissue may, therefore, facilitate smoother  
16 surgery. As there are several possible techniques for DMEK graft preparation, we  
17 studied few methods that are routinely used and compared them to find out the most  
18 suitable technique for DMEK graft preparation. Hence, we selected three different  
19 ways of stripping and 2 bubble techniques and compared multiple parameters.

20  
21 We observed that both, M4 and M5 are the techniques that use pressure between  
22 stroma and DM to create a bubble. Pneumatic dissection (M5) is carried out with full  
23 single force of air compared to SubHyS (M4) that utilized medium to high force of  
24 liquid depending on the primary small bubble. The pressure to obtain a complete  
25 bubble could have caused high cell death in M4 and M5 as it stretches the tissue. It  
26 was also observed that if the liquid is injected in the stroma, the force of the liquid  
27 cleaves the stroma and enters the stroma-DM plane to create a bubble. However, this  
28 phenomenon is not seen with air. The air is blocked in the stromal lamellae and as the  
29 density of air is less than liquid, it does not further cleave the posterior stroma and  
30 hence higher tissue wastage was noted in M5. Tissue wastage is significantly low in  
31 stripping techniques compared to bubble methods.

32  
33 Time required to produce a bubble from M5 was least if the bubble is generated from  
34 the first Pol. M4 is a controlled method hence requires more time than M5. However,  
35 it depends on the tissue and technique, if the DM to the stroma is tightly attached,  
36 the liquid can leak from the Pol. In such cases, the tissue should be immediately  
37 switched to a stripping technique. M3 requires longer time for graft preparation due  
38 to an extensive technique that requires punching, separating the graft and peeling  
39 compared with relatively easy methods such as M1 and M2.

40

1 Stripping methods have shown reduced cell death rate. All three stripping methods  
2 with different PIs were considered for this study. However, as M3 technique provides  
3 less endothelial damage, it could be worth investing the time but due to the expenses  
4 of this technique it may not be economically advantageous by many eye banks or  
5 surgeons with low volume grafts. Other techniques like M1 and M2 have also shown  
6 an acceptable range of cell death with both the methods having an economical  
7 advantage. The epithelial spatula or the Sinsky hook is not expensive and, therefore,  
8 if this technique is mastered, it can provide affordable and better quality DMEK grafts  
9 with minimal cell death.

10

11 For tightly attached tissues, a gentle oscillating stripping method can be used to strip  
12 the DMEK graft, however, for cases like previous cataract incision and horse shoe  
13 shaped tears, it is recommended that the PI should be from the opposite ends of the  
14 incision or rejecting the area of incision and initiating the stripping just after the  
15 incision. A para-central DMEK graft can be achieved this way (Parekh et al. 2017b).  
16 However, these kinds of tissues can only be stripped and cannot be bubbled as the  
17 bubble will pop out and the tissue will be torn or the liquid will leak from the incision  
18 site never allowing a bubble generation. There are several graft excision techniques  
19 that have been published in literature with advantages and disadvantages as recently  
20 reviewed and reported by Birbal R. et al (Birbal R. et al 2017). The limitation of this  
21 study was that we were only able to get data on five techniques that are routinely  
22 prepared by our eye bank and collaborators.

23

24 As stripping techniques have provided better graft quality compared to bubble  
25 techniques in this study and also from our previous experience (Parekh et al. 2017b),  
26 it is recommended to use a stripping method. M3 has shown to have the best graft  
27 quality, however, as the overall expenses and time of preparation for this method is  
28 significantly high for any low volume eye bank, it may have strict limitations. Stripping  
29 techniques with epithelial spatula and Sinsky hooks have served the best in terms of  
30 graft quality and economic feasibility. From this study, we have observed that M2,  
31 tissue preparation using Sinsky hook, could serve as the best graft preparation  
32 method considering all the parameters that include cell death, endothelial cell loss,  
33 time required to prepare the graft and costs. We believe that the techniques described  
34 in this article would help the surgeons and eye bankers to select the best option for  
35 DMEK graft preparation.

36

37

38

1 **ACKNOWLEDGEMENTS**

2 Funding/Support: None.

3

4 Financial Disclosures: Mohit Parekh, Davide Borroni, Alessandro Ruzza, Hannah J Levis,  
5 Stefano Ferrari, Diego Ponzin and Vito Romano have no financial interest to disclose.

6

7 Other Acknowledgements: None.

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

1 **REFERENCES**

2

3 Anshu A, Price MO & Price FW Jr (2012): Risk of corneal transplant rejection  
4 significantly reduced with Descemet's membrane endothelial keratoplasty.  
5 *Ophthalmology* **119**: 536-40.

6

7 Birbal RS, Sikder S, Lie JT, Groeneveld-van Beek EA, Oellerich S, Melles GRJ.  
8 Donor Tissue Preparation for Descemet Membrane Endothelial Keratoplasty:  
9 An Updated Review. *Cornea*. 2017 Oct 5. doi: 10.1097/ICO.0000000000001368.

10

11 Busin M, Scorcia V, Patel AK, et al (2010): Pneumatic dissection and storage of donor  
12 endothelial tissue for Descemet's membrane endothelial keratoplasty.  
13 *Ophthalmology* **117**: 1517-20.

14

15 Dapena I, Moutsouris K, Droutsas K, et al. (2011): Standardized "no-touch" technique  
16 for descemet membrane endothelial keratoplasty. *Arch Ophthalmol* **129**: 88-94.

17

18 Deng SX, Sanchez PJ, Chen L (2015): Clinical outcomes of Descemet membrane  
19 endothelial keratoplasty using eye bank prepared tissues. *Am J Ophthalmol* **159**: 590-  
20 96.

21

22 Gorovoy MS (2014): DMEK Complications. *Cornea* **33**: 101-4.

23

24 Groeneveld-van Beek EA, Lie JT, van der Wees J, Bruinsma M, Melles GR (2013):  
25 Standardized 'no-touch' donor tissue preparation for DALK and DMEK: harvesting  
26 undamaged anterior and posterior transplants from the same donor cornea. *Acta*  
27 *Ophthalmol* **91**: 145-50.

28

29 Kobayashi A, Murata N, Yokogawa H, et al (2015): Evaluation of internationally  
30 shipped prestripped donor tissue for descemet membrane endothelial keratoplasty  
31 by vital dye staining. *Cornea* **34**: 225-27.

32

33 Kruse FE, Laaser K, Cursiefen C, et al. (2011): A stepwise approach to donor  
34 preparation and insertion increases safety and outcome of Descemet membrane  
35 endothelial keratoplasty. *Cornea* **30**: 580-87.

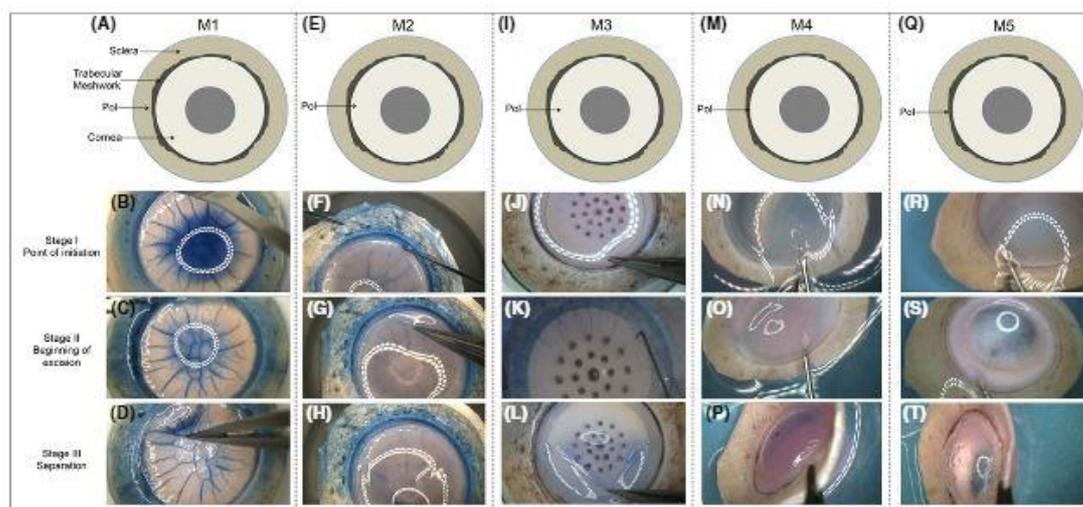
36

37 Melles GR, Ong TS, Ververs B, et al. (2006): Descemet Membrane Endothelial  
38 Keratoplasty (DMEK). *Cornea* **25**: 987-90.

39

## Parekh M. et al. DMEK graft preparation comparison

- 1 Parekh M, Ruzza A, Salvalaio G, et al. (2014): Descemet membrane endothelial  
2 keratoplasty tissue preparation from donor corneas using a standardized submerged  
3 hydro-separation method. *Am J Ophthalmol* **158**: 277-85.e1.  
4
- 5 Parekh M, Ruzza A, Ferrari S, et al. (2016): Preloaded Tissues for Descemet Membrane  
6 Endothelial Keratoplasty. *Am J Ophthalmol* **166**: 120-5.  
7
- 8 Parekh M, Leon P, Ruzza A, et al. (2017): Graft detachment and re-bubbling rate in  
9 Descemet Membrane Endothelial Keratoplasty. *Surv Ophthalmol* 2017 Jul 21. doi:  
10 10.1016/j.survophthal.2017.07.003. (A)
- 11 Parekh M, Baruzzo M, Favaro E, et al. (2017): Standardizing Descemet Membrane  
12 Endothelial Keratoplasty Graft Preparation Method in the Eye Bank-Experience of 527  
13 Descemet Membrane Endothelial Keratoplasty Tissues. *Cornea* **36**: 1458-1466. (B)
- 14 Price FW Jr, Price MO (2013): Evolution of endothelial keratoplasty. *Cornea* **32**: S28-  
15 S32.  
16
- 17 Ruzza A, Parekh M, Ferrari S, et al. (2015): Pre loaded donor corneal lenticules in a  
18 new validated 3D printed smart storage glide for Descemet stripping automated  
19 endothelial keratoplasty. *Br J Ophthalmol* **99**: 1388–95.  
20
- 21 Terry MA (2012): Endothelial keratoplasty: why aren't we all doing Descemet  
22 membrane endothelial keratoplasty?. *Cornea* **31**: 469-71.  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39 **FIGURES**



1

2 **Figure 1:** Schematic representation, stage I, II and III of different DMEK graft  
3 preparation methods.

4 M1 represents the technique that uses an epithelial spatula to swipe the DMEK graft  
5 all the way from the trabecular meshwork (TM). A) Point of Initiation (Pol) is shown  
6 just before the TM, B) Pol on a donor cornea, C) Excision begins after removing the  
7 TM at the periphery of the DMEK graft and D) Separation of a DMEK graft from the  
8 underlying stroma is carried out using acute forceps using stripping method.

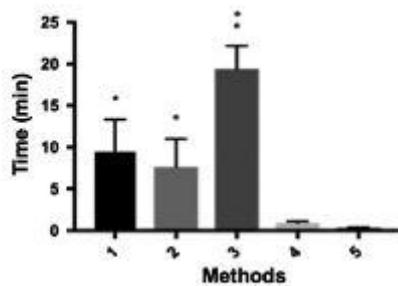
9 M2 represents the technique that uses a Sinsky hook to score the peripheral  
10 endothelium. E) Pol is shown at the periphery of the endothelium just before the TM  
11 on the corneal side. F) Pol near the TM, the donor endothelium is scored using a  
12 Sinsky hook along the circumference of the cornea and G) Separated from the stroma  
13 followed by H) excision of DMEK graft using acute forceps using stripping method.

14 M3 represents the technique that uses a donor punch. I) Pol 9.5mm from the central  
15 cornea, J) Donor punch that is used for superficial tapping on the endothelial side, K)  
16 Cleavage hook used to separate the DMEK graft from the trephined site and L) Acute  
17 forceps used to strip and excise the DMEK graft from the cornea.

18 M4 represents the submerged hydro-separation technique. M) Pol is just beneath the  
19 TM, N) A needle of the syringe is advanced in the stroma-DM plane until the bevel is  
20 completely inside, O) with moderate to high pressure, the liquid is forced in the plane  
21 to create a liquid bubble, P) a full length liquid bubble separating the stroma with DM.

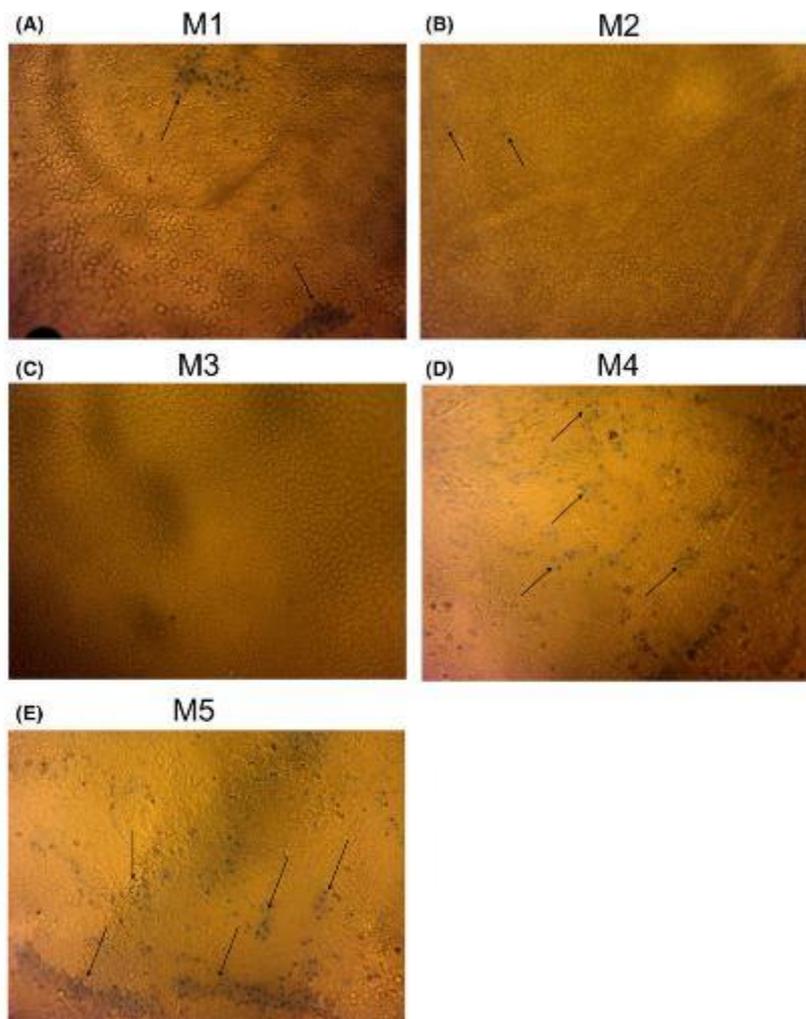
22 M5 represents the pneumatic dissection technique. Q) Pol is from the sclera just  
23 outside the TM, R) a 25G needle of the syringe is advanced tangentially, S) an air  
24 bubble is initiated with full force, if there is a central air bubble with stromal lamellae  
25 filled with air, the Pol can be changed to another site and the bubble is generated, T)  
26 A full length air bubble is thus created.

27 Note: The circle with white dots is an impression originating from the LED light fixed  
28 on the microscope.



1

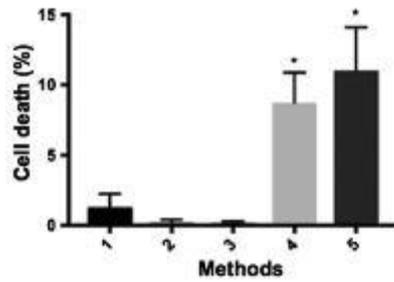
2 **Figure 2:** Time required to prepare the graft in each method was statistically  
3 significant with M3 taking highest time to prepare a graft. \*Methods take more time  
4 than others. \*\*Method is the slowest of all.



5

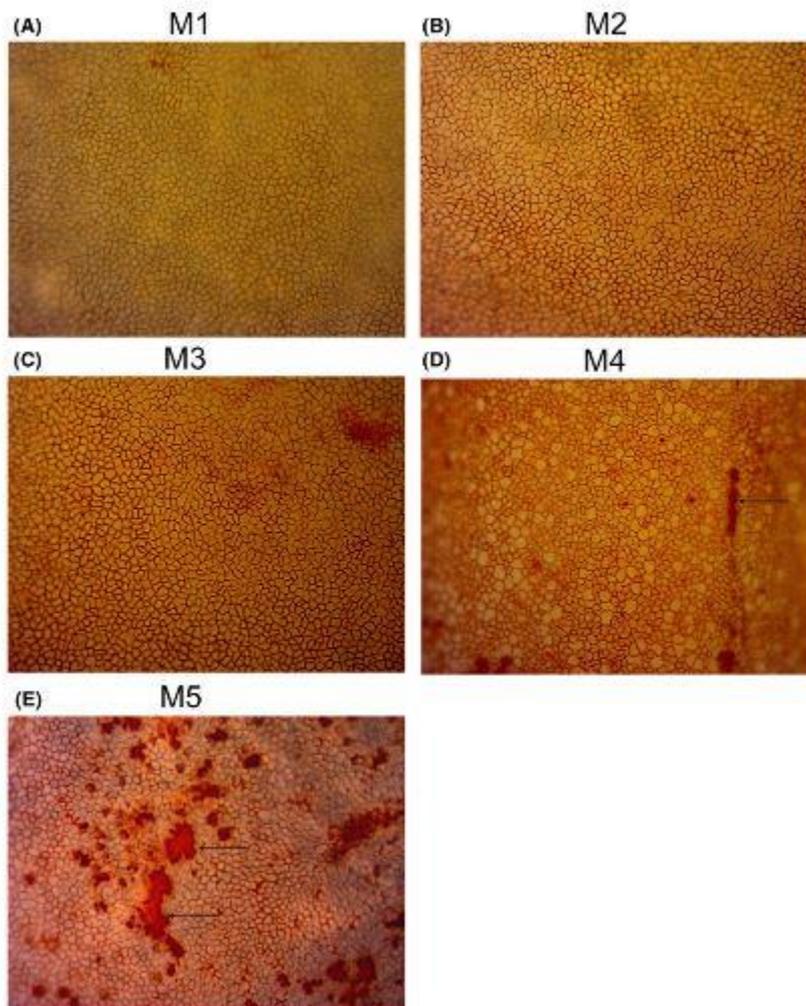
6 **Figure 3:** Endothelial cell density and cell death observed using trypan blue positive  
7 cells (marked with arrow). A) M1 showed acceptable range of cell death, B) M2  
8 showed minimal amount of cell death, C) M3 did not show any cell death, D) M4 and  
9 E) M5 showed critical levels of cell death.

10



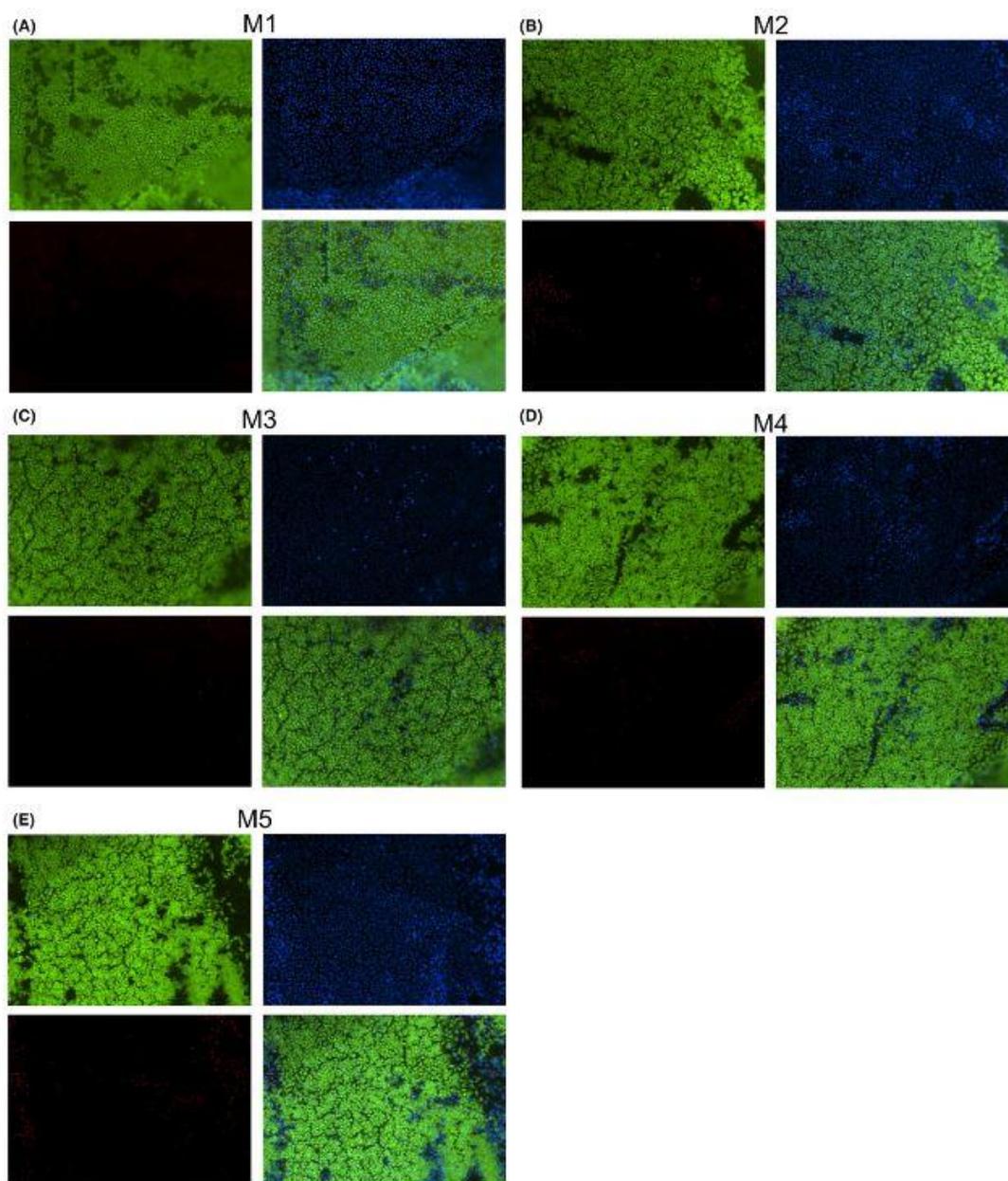
1

2 **Figure 4:** Endothelial cell death was statistically significant ( $p < 0.05$ ) between all the  
3 methods. \*Methods cause significant cell death compared to others.

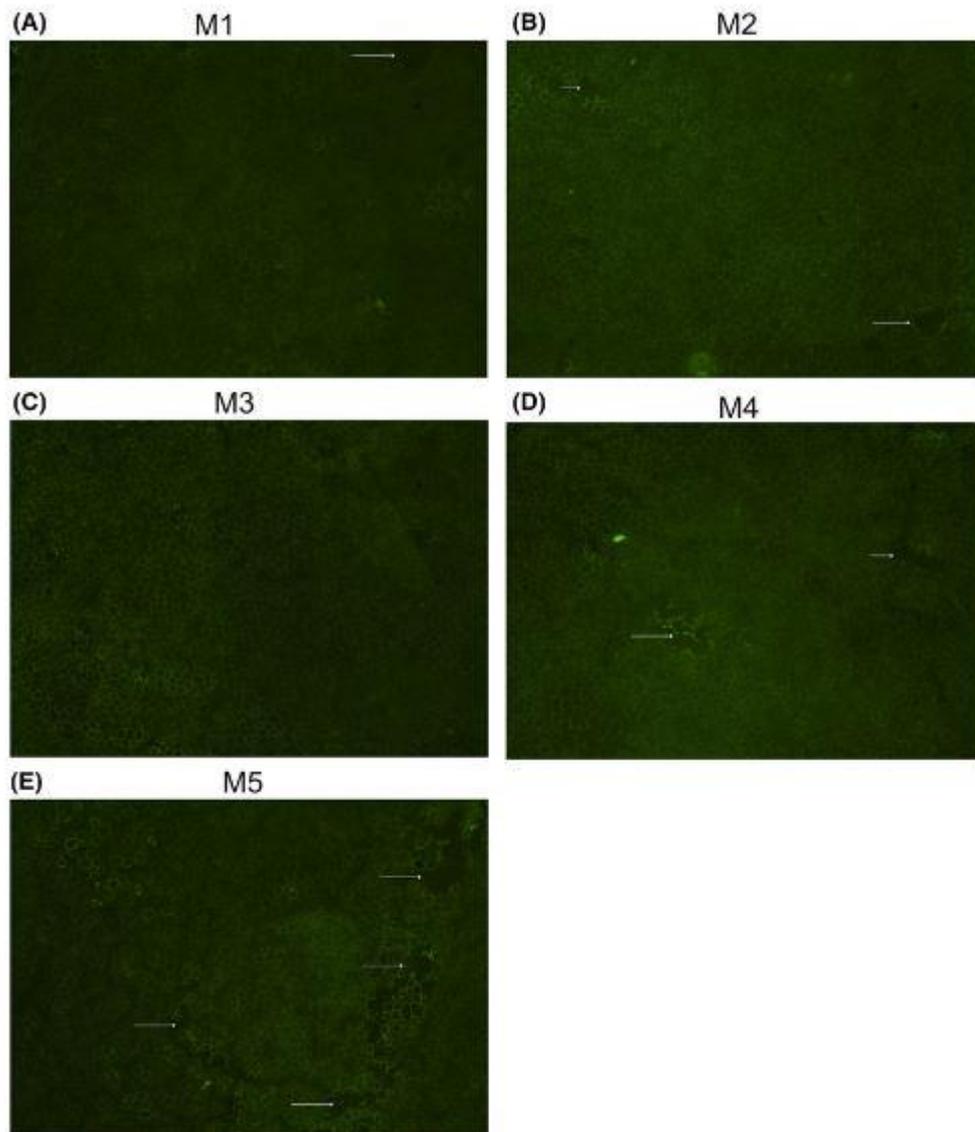


4

5 **Figure 5:** Alizarin red staining for morphological analysis showed A) M1, B) M2 and C)  
6 M3 that did not differ in morphology however, D) M4 showed pleomorphic cells and  
7 areas without cells (marked with arrow) and E) M5 showed pleomorphic cells and  
8 severe amount of denuded areas (marked with arrows). Scale: 100X magnification.



1  
2 **Figure 6:** Hoechst, Ethidium homodimer and Calcein AM (HEC) staining to determine  
3 live/dead cells. Live cells were determined by green (Calcein AM staining), nuclei of  
4 the cells were determined by blue (Hoechst staining) and the dead cells were  
5 determined with red (Ethidium homodimer staining) color. All the results were  
6 correlated with trypan blue staining. It was observed that A) M1 and B) M2 showed  
7 acceptable cell death but C) M3 did not show any cell death whereas D) M4 and E) M5  
8 showed critical levels of dead cells. There were some denuded areas also found in M4  
9 and M5.



1  
2 **Figure 7:** Zonula Occludens-1 expression was seen in all the methods wherever the  
3 cells were present. A-E) There were denuded areas as observed earlier and ZO-1 was  
4 not expressed in those areas. The morphology and hexagonality was observed and  
5 normal to pleomorphic cells in all the cases were found. Scale: 100X magnification.

1 **TABLES**

2

	Age (Years)	Male/Female	Post mortem (Hours)	ECD (cells/mm <sup>2</sup> )
<b>M1</b>	75.0(3.3)	6/1	16.8(5.3)	2042(113.3)
<b>M2</b>	73.3(2.5)	4/3	12.5(4.1)	1971(48.8)
<b>M3</b>	73.0(4.1)	5/2	10.7(6.9)	1975(95.7)
<b>M4</b>	65.8(10.4)	5/2	13.7(6.3)	1960(54.8)
<b>M5</b>	72.1(3.9)	4/3	10.1(3.6)	2042(78.7)

3

4 **Table 1:** Donor characteristics in terms of age, gender, post mortem hours and  
 5 endothelial cell density from corneas used in each methods M1-M5. All the data are  
 6 presented as Mean(SD) except the data on gender. ECD stands for Endothelial Cell  
 7 Density.

8

	Dead cells (%) Mean (SD) [Range]	ECD (cells/mm <sup>2</sup> ) Mean (SD) [Range]	ECL (%) Mean (SD) [Range]	Time (minutes) Mean (SD) [Range]
<b>M1</b>	1.29(0.96) [0.3-3]	1928(75) [1800-2000]	2.71(4.99) [0-10]	9.43(3.91) [4.5-15]
<b>M2</b>	0.21(0.17) [0-0.5]	1921(90) [1800-2000]	2.96(7.42) [0-14.2]	7.64(3.37) [4.5-14]
<b>M3</b>	0.13(0.14) [0-0.4]	1957(97) [1800-2100]	1.18(7.37) [0-4.76]	19.41(2.77) [15-23.5]
<b>M4</b>	8.71(2.14) [8-12]	1914(89) [1800-2000]	3.34(7.25) [0-10]	0.27(0.14) [0.1-0.4]
<b>M5</b>	11.00(3.11) [8-16]	1900(81) [1800-2000]	4.06(7.06) [5-10]	0.17(0.16) [0.7-0.2]
<b>pValue</b>	<b>&lt;0.05*</b>	0.96	0.96	<b>&lt;0.05*</b>

9

10 **Table 2:** Comparison between different graft preparation methods: 1° column cell  
 11 death evaluated with trypan blue, 2° column ECD evaluated with light microscope, 3°  
 12 column overall endothelial cell loss, 4° column time evaluated with stopwatch for each  
 13 method.

14 Key: ECD – Endothelial Cell density; ECL – Endothelial Cell Loss; \*Statistically significant  
 15 values

	Instrument	Diameter of graft obtained (mm)	Risk of peripheral tears	Risk of bubble burst	Suction base	Forceps*	Costs** Approx (USD)
<b>M1</b>	Epithelial Spatula	11	High	Not applicable	Not mandatory	Medium forceps (J3683, e.Janach) size 3, straight, pointed tips, 120 mm	8
<b>M2</b>	Sinsky Hook	10.5	High	Not applicable	Not mandatory	Medium forceps (J3683, e.Janach) size 3, straight, pointed tips, 120 mm	10
<b>M3</b>	Donor Trephine	8.25-10 (Wide range)	Moderate	Not applicable	Mandatory	Medium forceps (J3683, e.Janach) size 3, straight, pointed tips, 120 mm	150.0
<b>M4</b>	25G needle with 1 mL syringe	11	Not applicable	Moderate	Not required	Not applicable	<1
<b>M5</b>	25G needle with 1 mL syringe	11	Not applicable	Moderate-High	Not required	Not applicable	<1

1

2

3 **Table 3:** Parameters considered: instruments used, diameter of the graft, risk of  
 4 peripheral tears or bubble burst, inclusion of suction base, forceps and overall costs.

5 \*Forceps – depends on the surgeon. We prefer acute forceps as they grasp sufficient  
 6 tissue required for peeling and to reduce overall endothelial cell damage.

7 \*\*Costs – the overall costs are mainly for the instruments that are used. As these costs  
 8 are the primary costs that an eye bank or the surgeon will have to pay, we considered  
 9 this as our point of reference. This mainly includes the price of the instrument (Column  
 10 one).

11

1 **SUPPLEMENTARY VIDEO LEGENDS**

2

3 **Supplementary video 1:** Stripping from the trabecular meshwork – Epithelial spatula  
4 was used to swipe the trabecular meshwork (TM) towards the cornea to release the  
5 peripheral endothelium. The TM was cut and using trypan blue as visualizing dye, the  
6 Descemet membrane endothelial keratoplasty (DMEK) tissue was peeled.

7

8 **Supplementary video 2:** Stripping by scoring the peripheral endothelium – Sinsky  
9 hook was used to score the peripheral endothelium and the DMEK was peeled from  
10 the scored area.

11

12 **Supplementary video 3:** Stripping by punch method – A 9.5mm donor punch was used  
13 to superficially trephine the endothelium. Cleavage hook was used to identify the  
14 cleavage plane followed by stripping the tissue.

15

16 **Supplementary video 4:** Submerged Hydro-Separation (SubHyS) Method – 25G  
17 needle attached to a 1 mL syringe filled with tissue culture medium was injected  
18 between the stroma and Descemet membrane. Medium to high pressure was used to  
19 create a liquid bubble.

20

21 **Supplementary video 5:** Pneumatic dissection method – 25G needle attached to 1 mL  
22 syringe filled with air was injected between the stroma and Descemet membrane. Full  
23 pressure was applied to create an air bubble.

24

25