



Evaluation of the Effect of Cavity Preparation Using Er,Cr:YSGG Laser on Microleakage of Class V Composite Restorations

Zahra Khamverdi^{1*}, Loghman Rezaei-Soufi², Hamidreza Haseli Paik³, Puya Jabari³, Mohammad Ahmadian⁴

Abstract

Background: The application of laser in dentistry for medical purposes such as caries removal, preparation of restorative cavities, and dental surface treatment for more effective bonding of restorative materials to the tooth has been significant. The present experimental study aimed to evaluate the effect of cavity preparation on microleakage by using erbium, chromium-doped yttrium, scandium, gallium, and garnet (Er,Cr:YSGG) lasers, and to compare it with the effect of bur on microleakage in class V composite restorations.

Methods: In this experimental study, 20 intact human premolar teeth were randomly divided into 2 equal groups according to the cavity preparation technique: G1: laser cavity preparation (LCP) using a Er,Cr:YSGG laser (Bio Lase, USA), and G2: bur cavity preparation (BCP). Standard class V cavity was prepared on both lingual and buccal surfaces in two groups. The samples underwent thermocycling for 3000 times (5-55°C) and were immersed in a methylene blue 2% solution for 24 hours. After buccolingual sectioning from the middle of the restoration, a stereomicroscope with 20 x magnification was used to measure the penetration rate of the dye and to determine the score for microleakage. Data were analyzed using SPSS (version 16) software and Mann-Whitney U test ($\alpha=5\%$).

Results: According to the study results, the minimum and maximum microleakage values were observed in the occlusal and gingival margins, respectively, which were identically for both groups. Comparing two groups (BCP and LCP) revealed that there was no significant difference between them in terms of microleakage values at the occlusal and gingival margins ($P>0.05$).

Conclusions: It was concluded that cavity preparation using Er,Cr:YSGG laser had microleakage values similar to those found with conventional cavity preparation (bur) method in class V composite restorations.

*Correspondence to

Zahra Khamverdi,
Tel: +98 9183122095,
Email: z.khamverdi95@gmail.com

Keywords: Microleakage, Er,Cr:YSGG laser, Bur, Class V composite restoration

Received March 24, 2021
Accepted April 24, 2021
ePublished September 29, 2021



Citation: Khamverdi Z, Rezaei-Soufi L, Haseli Paik H, Jabari P, Ahmadian M. Evaluation of the effect of cavity preparation using Er,Cr:YSGG laser on microleakage of class V composite restorations. Avicenna J Dent Res. 2021;13(3):81-85. doi: 10.34172/ajdr.2021.16.

Background

The use of laser in dentistry has been increased in recent years; and this increase, in turn, has brought it to dentists' attention. The laser is used in restorative dentistry for various processes such as cavity preparation, reduction of dentinal sensitivity, tooth whitening, polymerization of composite resins, and treatment of organic and inorganic dental structures (1,2).

Erbium lasers are among the most commonly used lasers in dentistry. They bring several benefits to dentistry including minimal vibration, reduction of noise, and minimal need to anesthesia compared to conventional systems for cavity preparation and caries excavation (3). Er,Cr:YSGG is a new laser type with a wavelength of 2780 nm, and has the highest absorption

Highlights

- ▶ Cavity preparation using Er,Cr: YSGG laser has microleakage values similar to those found with conventional cavity preparation (bur) method in class V composite restorations.

property in hydroxyapatite crystals (4). Combining this laser with a hydrokinetic system creates a cooling effect and minimizes thermal damage to the pulp (5). The mechanism of this laser operates when it is irradiated and its energy is absorbed in the surface of the tissue and, then, it evaporates water and expands the crystals. This mechanism can be used for dental operations such as cutting of enamel, dentin, and bone (6).

Microleakage can cause problems such as secondary

¹Professor, Dental Research Center, Department of Restorative Dentistry, Dental School, Hamadan University of Medical Sciences, Hamadan, Iran. ²Associated Professor, Department of Restorative Dentistry, Dental School, Hamadan University of Medical Sciences, Hamadan, Iran. ³Dentist. ⁴Student Research Committee, Hamadan University of Medical Sciences, Hamadan, Iran

caries, tooth sensitivity, pulpal irritation, and pulpal necrosis. It commonly occurs in the gingival margin of the restorations where it is bonded to the dentin due to the absence of enamel. The bonding of composite to the dentin often creates problems in the restorations' gingival margin which is the result of the low surface energy and the presence of dentinal tubular fluid. As a result, composite restorations are separated from the cavity wall and a gap is formed after polymerization shrinkage (7). Therefore, it is important to propose a method that can reduce microleakage and control progression of caries in these areas. The application of lasers in restorative dentistry has been developed to such an extent that some practitioners have considered it as an alternative method to conventional drilling and acid etching techniques (6-8).

Previous studies have evaluated the microleakage of composite resin restorations in laser cavity preparation (LCP) and bur cavity preparation (BCP) groups, but they have failed to yield conclusive results. Some studies have reported that LCP is more effective than BCP, whereas other studies have not differentiated between them (9).

The present study, therefore, aimed to evaluate the effect of cavity preparation with assisted-Er,Cr:YSGG laser, and compare it with the effect of BCP on the microleakage of class V composite restorations.

Materials and Methods

This study was an experimental study carried out in the Laboratory of Dental Materials and Laser for Dental Research Center of Hamadan University of Medical Sciences. According to Cochran's formula, the required sample size for this study was determined to be 20 teeth. Extracted human teeth without cracks, restoration, abrasion, decay, and defects were included in the study.

Twenty healthy human premolars which had been extracted due to orthodontic treatment purposes were selected and kept in 10% formalin solution. Then, the teeth were cleaned for removing debris and stored in normal saline. The teeth were placed in the distilled water at room temperature for 24 hours before experiment. The samples were randomized into two equal groups by balanced block randomization.

As for group 1, two cavities were prepared on buccal and lingual surfaces of each tooth using Er,Cr:YSGG laser (Bio Lase, USA). A standard class V cavity with dimensions of 2 mm mesiodistal width, 3 mm height, and 3 mm depth, as well as a 1 mm bevel in the occlusal margin on enamel was prepared with the following settings:

To cut the enamel: laser frequency 20 Hz, laser mode H-mode, power 5.5 W, pulse energy 275 mJ/pulse, 95% air flow and 80% water flow.

To cut the dentine: laser frequency 20 Hz, laser mode H-mode, power 3.5 W, pulse energy 275 mJ/pulse, 95% air flow and 80% water flow.

As for group 2, standard class V cavities were prepared

with similar dimensions on the buccal and lingual surfaces of each tooth using a high speed handpiece and cylindrical diamond bur (#835, Diatech, Scissdental, Switzerland) with water and air cooling. The bur was replaced after drilling five cavities. The cavities in two groups were etched completely using 37% phosphoric acid gel (Etch, Ultradent, South Jordan, USA Ultra) for 15 seconds, and then they were washed with water for 10 seconds and, finally, were dried. Two layers of five generation light-cured bonding adhesive (Single Bond, 3M, ESPE, USA) were applied to the cavity for 10 seconds using micro-brush and, then, they were dried with gentle air for 5 seconds and cured for 10 seconds. A light-cure composite (Z250, 3M ESPE, MN, USA) was layered incrementally according to manufacturer's instructions in the cavity, and each layer was light-cured for 20 seconds (APOZA, Woodpecker, Guangxi, China) with the intensity of 600 mW/cm². Finishing and polishing stages were then performed for both groups using polishing burs and discs (Soflex, St. Paul, MN, USA, 3M, ESPSE). The samples were kept in distilled water for one week to complete the polymerization. The samples underwent thermocycling for 3000 times between 5°C-55°C for 30 seconds. Following the thermal cycles and in order for measuring the microleakage, the apex of the teeth was sealed with adhesive wax and the teeth surfaces were covered with a nail lacquer layer, one millimeter adjacent to the restoration. The samples were immersed in a 2% methylene blue solution for 24 hours and rinsed under running water for 2 minutes; then the samples mounted in self-curing acrylic (Kaveh, Tehran, Iran) after drying. The teeth were sectioned buccolingually from the middle part of restoration with a nonstop machine (I12207 model, Albany, New York, USA). The samples were assessed by a stereomicroscope (Olympus, Tokyo, Japan) at 20X magnification to measure the dye penetration and to determine the microleakage score. The samples were scored at occlusal and gingival margins according to dye penetration as follows:

Class 0=No color penetration observed.

Class 1=Dye penetration to 1/3 depth of the cavity.

Class 2=Dye penetration to 2/3 depth of the cavity.

Class 3=Dye penetration throughout the cavity depth.

Data were analyzed using SPSS software (version 16), and descriptive statistics including the frequency and percent for categorical data, the mean and SD for continuous data, as well as the variables were all examined and calculated. Mann-Whitney U test was used for comparing study groups. The significant level was considered as being 0.05.

Results

The results for group LCP showed that the occlusal margin had a lower microleakage so that 85% of the samples were placed in class 0, whereas this value was 55% for the gingival margin. Similarly, the frequency of BCP samples had the least value of microleakage at the occlusal margin

(class 0=60%). The percentages of microleakage related to LCP and BCP groups at occlusal and gingival margins are summarized in Table 1.

Comparing LCP and BCP groups in terms of microleakage demonstrated that the least amount of microleakage in the occlusal and gingival margins occurred in the laser group. Mann-Whitney U test showed that there was no significant difference between LCP and BCP groups regarding the score of microleakage at the occlusal margin. In addition, there was no significant difference between drilling and laser cutting methods in terms of the gingival margin ($P>0.05$). Overall, no significantly difference was found between two groups concerning the microleakage scores ($P=0.192$) (Table 2).

Discussion

Pulp reactions to restorative materials mainly depend on the degree of microleakage. Bacteria can survive and reproduce in the gap between composite and cavity wall where it is filled with oral fluids. Therefore, restoration marginal seal takes on an added importance for durability of adhesive restorations, especially the composites. Lack of seal at the margins allows the entrance of bacteria and fluids, causing post-treatment sensitivity, the pathological response of pulp, and secondary caries (10).

Microleakage occurs when bacteria and their toxins

pass through the restoration edges and cavity walls due to the temperature alterations in the oral environment, the mechanical fatigue caused by periodic chewing forces and, most importantly, the orientation of polymerization shrinkage towards the stronger tooth-composite bonding surface (11,12).

In restorative dentistry, lasers are used for achieving goals such as enamel reinforcement, enamel and dentin surface conditioning, as well as cavity preparation. Some researchers have proposed using lasers as a viable alternative to conventional methods of BCP and etching (8,13).

Few studies have investigated the microleakage of composite resin materials in restorations following LCP compared to BCP. Some studies have determined that LCP is more effective method than the BCP, while others have not differentiated between the two methods. Due to the lack of sufficient data, therefore, the present study aimed to investigate the possible advantages of assisted-Er,Cr:YSGG LCP, and to compare it with BCP regarding the microleakage of class V composite restorations.

During the preparation of the cavity using laser, the lack of control over the pulp temperature causes pulpal damage. This complication has limited the use of laser in operative dentistry (14). Er,Cr:YSGG laser generates a maximum of 2°C, which causes the least amount of damage to the pulp and surrounding tissues. Since it has high absorption in water and is strongly absorbed by hydroxyl radicals in the hydroxyapatite structure, it is useful for cutting enamel (15). It has been reported the thermal changes occurring in the mouth are small; so the actual results are obtained when the samples are exposed to the thermal spectrum in a short time. Between the two baths, therefore, sufficient intervals are provided for samples to return to body temperature (16).

Thermal changes are among the essential factors that can affect the marginal seal due to the difference in the thermal expansion coefficient between the tooth and the composite. Thus the use of thermal cycles is necessary to simulate the oral environment and to increase the validity of the results (17). Thermocycling uses two temperature ranges, with the upper limit being 45-60°C and the lower limit as 4-15°C. It is also recommended that the specimens

Table 1. Leakage Score Values in Groups LCP and BCP in Both Gingival and Occlusal Margins

	Class of Microleakage	Occlusal Margin	Gingival Margin	Total
LCP	0	17 (85%)	11 (55%)	28 (70%)
	1	2 (10%)	6 (30%)	8 (20%)
	2	0 (0%)	3 (15%)	3 (7.5%)
	3	1 (5%)	0 (0%)	1 (2.5%)
Total		20 (100%)	20 (100%)	40 (100%)
BCP	0	14 (70%)	10 (50%)	24 (60%)
	1	3 (15%)	3 (15%)	5 (15%)
	2	0 (0%)	4 (20%)	4 (10%)
	3	3 (15%)	3 (15%)	6 (15%)
Total		20 (100%)	20 (100%)	40 (100%)

Table 2. Comparing Two Groups (i.e., LCP and BCP) Regarding Microleakage Using the Mann-Whitney U test

	Group	Class of Microleakage				Test Statistics	P Value
		0	1	2	3		
Occlusal margin	LCP	17	2	0	1	-1.17	0.397
	BCP	14	3	0	3		
Gingival margin	LCP	11	6	3	0	-0.916	0.414
	BCP	10	3	4	3		
Total	LCP	28	8	3	1	-1.31	0.192
	BCP	24	6	4	6		

be immersed in the hot and cold baths for 10, 15, 30, 60 and 120 seconds (18).

During the thermal cycling stages and due to the differences in the thermal expansion coefficient of restoration and tooth structure, stress develops at the borders between the tooth and the restoration. According to ISO TR 11450 (1994), samples must go through a heat cycle of 5-55°C at least 500 times. But some researchers have revealed that this number of heat cycles are too small to approach oral conditions, and they have recommended that the cycle be repeated 2000-3000 times (19). In this study, The samples were subjected to 3000 thermal cycles between 5 and 55°C for 30 seconds in this study in order for simulating the oral environment of the specimens (4).

Dye penetration is the most commonly used technique for assessing microleakage. The Methylene blue 2% is cost-effective, easy-to-use, and safe, and does not need proprietary tools to investigate; therefore, a stereomicroscope can easily measure the amount of microleakage (19).

Ernst et al (20) showed that 30 minutes of dye penetration was sufficient time to characterize the marginal seal. However, most studies have found 24 to 48 hours as required time to determine the microleakage. In this study, the dye penetration time was 24 hours (14).

Class V composite restorations have no macro-mechanical adhesion. Therefore, marginal integrity is not be affected by this type of retention. The restoration occlusal and gingival margins are located in enamel and dentin, respectively, and can be examined. Thus, laboratory studies of microleakage are usually performed on class V restorations (21).

According to our study results, there was the least amount of microleakage in the occlusal and gingival margins in the LCP group. The amount of microleakage in the occlusal and gingival margin was not significantly different between the two methods. This finding could be attributed to the effect that this laser exerted on the surface features of the cavity. The Er,Cr:YSGG laser appeared to have caused surface roughness in a different form (shell-like) by removing the smear layer, and thereby providing suitable conditions for a more consistent fitness to the cavity surface (14).

Yazici et al (14) demonstrated that there was no statistically significant difference in microleakage between the LCP and BCP groups, which was in agreement with our study finding. In another study by Gutknecht et al (8), no significant difference was observed between the LCP and BCP, which was also in line with our study results.

According to the findings from a study by Subramaniam and Pandey (15), our study results could be generalized to primary teeth. Subramaniam and Pandey investigated the effect of Er,Cr:YSGG laser on microleakage of Class III composite restorations of primary teeth, and found no significant difference between the amounts of microleakage

in two methods adopted for cavity preparation.

The matching of the results from the above-mentioned study and those from our study was attributed to the similar conditions existed in both studies, such as the power and intensity of laser weathering, the type of composite filling technique, etc. Our study results contradicted the findings from Fattah et al (22) since they had reported a significant difference between the studied groups concerning the microleakage. It was claimed that compared to the BCP, the application of laser combination with 37% phosphoric for etching decreased the leakage significantly.

Shahabi et al (6) also reported a statistically significant difference between their study groups, which was inconsistent with our results. According to the findings from a study by Hossain et al (23), the microleakage in the LCP was less than that in the BCP, which was also inconsistent with our study results.

The inconsistencies between the results from the given studies and those from ours could be associated with the different conditions existed in each study, such as the power applied to the laser, the amount of laser weathering, the cavity's margin features, the type of composite, the bonding material used, filling method, curing duration, number of heat cycles, and storage phase duration (6,22,23).

In the study by Ertürk Avunduk and Bağlar et al (24), microleakage in four groups of different caries-removing methods including BCP, Carisolv gel, Papacarie gel, and LCP was investigated. The samples were assessed by light microscopy and scanning electron microscopy (SEM). The results demonstrated no significant difference between the groups regarding the microleakage in dentinal zones, but a significant difference was observed between the Papacarie gel and LCP groups concerning the enamel zones.

However, according to the mentioned advantages of lasers in dental procedures such as having no noise, absence of vibration, and not causing pain, the application of Er,Cr:YSGG laser for cavity preparation is indicated in children, elderly and anyone with phobias of dental procedures. Given the fact that the use of Er,Cr:YSGG lasers has not received sufficient attention so far, our study findings can serve as an essential background for further investigations. In other words, revealing other aspects of the Er,Cr:YSGG laser utilization such as the use of different restorative materials, laser preparation techniques, and types of teeth entails conducting further studies to produce reliable results on its utilization. Taking into account the laboratory nature of the present study, it is recommended that similar studies be carried out at the clinical level in order to simulate oral cavity circumstances and increase the validity of the results.

Conclusions

Given the limitations of this study, it was concluded that cavity preparation using Er,Cr:YSGG laser had

microleakage values similar to those found in conventional cavity preparation (bur) in class V composite restorations.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

Ethical Statement

The present study was approved by the Research and Medical Ethics Committee of Hamadan University of Medical Sciences (ethics code: IR.UMSHA.REC.1397.65).

Authors' Contribution

Study concept and design: ZK, HHP; acquisition of data: HHP; analysis and interpretation of data: LRS; drafting of the manuscript: All authors; manuscript editing: Zahra Khamverdi, MA, PJ; study supervision: ZK.

Funding

This study was funded by Hamadan University of Medical Sciences.

References

- Celik C, Ozel Y, Bağış B, Erkut S. Effect of laser irradiation and cavity disinfectant application on the microtensile bond strength of different adhesive systems. *Photomed Laser Surg.* 2010;28(2):267-72. doi: [10.1089/pho.2009.2509](https://doi.org/10.1089/pho.2009.2509).
- Hashim NT, Gasmalla BG, Sabahelkheir AH, Awooda AM. Effect of the clinical application of the diode laser (810 nm) in the treatment of dentine hypersensitivity. *BMC Res Notes.* 2014;7:31. doi: [10.1186/1756-0500-7-31](https://doi.org/10.1186/1756-0500-7-31).
- Guyen Y, Aktoren O. Shear bond strength and ultrastructural interface analysis of different adhesive systems to Er:YAG laser-prepared dentin. *Lasers Med Sci.* 2015;30(2):769-78. doi: [10.1007/s10103-013-1424-0](https://doi.org/10.1007/s10103-013-1424-0).
- Moldes VL, Capp CI, Navarro RS, Matos AB, Youssef MN, Cassoni A. In vitro microleakage of composite restorations prepared by Er:YAG/Er,Cr:YSGG lasers and conventional drills associated with two adhesive systems. *J Adhes Dent.* 2009;11(3):221-9.
- Shirani F, Birang R, Malekipour MR, Hourmehr Z, Kazemi S. Shear bond strength of resin composite bonded with two adhesives: influence of Er:YAG laser irradiation distance. *Dent Res J (Isfahan).* 2014;11(6):689-94.
- Shahabi S, Chiniforush N, Fekrazad R, Fatemi SM. Comparison of tensile bond strength of composite to dentin in conventional or laser-prepared cavities (Er,Cr:YSGG). *J Oral Laser Appl.* 2010;10(2-3):107-10.
- Demarco FE, Ramos OL, Mota CS, Formolo E, Justino LM. Influence of different restorative techniques on microleakage in class II cavities with gingival wall in cementum. *Oper Dent.* 2001;26(3):253-9.
- Gutknecht N, Apel C, Schäfer C, Lampert F. Microleakage of composite fillings in Er,Cr:YSGG laser-prepared class II cavities. *Lasers Surg Med.* 2001;28(4):371-4. doi: [10.1002/lsm.1064](https://doi.org/10.1002/lsm.1064).
- Aranha AC, Turbino ML, Powell GL, de Paula Eduardo C. Assessing microleakage of class V resin composite restorations after Er:YAG laser and bur preparation. *Lasers Surg Med.* 2005;37(2):172-7. doi: [10.1002/lsm.20208](https://doi.org/10.1002/lsm.20208).
- Yavuz I, Aydin AH. New method for measurement of surface areas of microleakage at the primary teeth by biomolecule characteristics of methylene blue. *Biotechnol Biotechnol Equip.* 2005;19(1):181-7. doi: [10.1080/13102818.2005.10817178](https://doi.org/10.1080/13102818.2005.10817178).
- El-Mowafy O, El-Badrawy W, Eltanty A, Abbasi K, Habib N. Gingival microleakage of class II resin composite restorations with fiber inserts. *Oper Dent.* 2007;32(3):298-305. doi: [10.2341/06-86](https://doi.org/10.2341/06-86).
- Kubo S, Yokota H, Sata Y, Hayashi Y. The effect of flexural load cycling on the microleakage of cervical resin composites. *Oper Dent.* 2001;26(5):451-9.
- Hibst R, Keller U. Experimental studies of the application of the Er:YAG laser on dental hard substances: I. Measurement of the ablation rate. *Lasers Surg Med.* 1989;9(4):338-44. doi: [10.1002/lsm.1900090405](https://doi.org/10.1002/lsm.1900090405).
- Yazici AR, Yıldırım Z, Antonson SA, Kilinc E, Koch D, Antonson DE, et al. Comparison of the Er,Cr:YSGG laser with a chemical vapour deposition bur and conventional techniques for cavity preparation: a microleakage study. *Lasers Med Sci.* 2012;27(1):23-9. doi: [10.1007/s10103-010-0833-6](https://doi.org/10.1007/s10103-010-0833-6).
- Subramaniam P, Pandey A. Assessment of microleakage of a composite resin restoration in primary teeth following class III cavity preparation using Er,Cr:YSGG laser: an in vitro study. *J Lasers Med Sci.* 2016;7(3):172-6. doi: [10.15171/jlms.2016.30](https://doi.org/10.15171/jlms.2016.30).
- Alani AH, Toh CG. Detection of microleakage around dental restorations: a review. *Oper Dent.* 1997;22(4):173-85.
- Stanford SK, Wozniak WT, Fan PL. The need for standardization of test protocols. *Semin Orthod.* 1997;3(3):206-9. doi: [10.1016/s1073-8746\(97\)80071-0](https://doi.org/10.1016/s1073-8746(97)80071-0).
- Grieve AR, Saunders WP, Alani AH. The effects of dentine bonding agents on marginal leakage of composite restorations--long-term studies. *J Oral Rehabil.* 1993;20(1):11-8. doi: [10.1111/j.1365-2842.1993.tb01510.x](https://doi.org/10.1111/j.1365-2842.1993.tb01510.x).
- Attin T, Hannig C, Wiegand A, Attin R. Effect of bleaching on restorative materials and restorations--a systematic review. *Dent Mater.* 2004;20(9):852-61. doi: [10.1016/j.dental.2004.04.002](https://doi.org/10.1016/j.dental.2004.04.002).
- Ernst CP, Galler P, Willershausen B, Haller B. Marginal integrity of class V restorations: SEM versus dye penetration. *Dent Mater.* 2008;24(3):319-27. doi: [10.1016/j.dental.2007.06.002](https://doi.org/10.1016/j.dental.2007.06.002).
- De Munck J, Van Landuyt K, Peumans M, Poitevin A, Lambrechts P, Braem M, et al. A critical review of the durability of adhesion to tooth tissue: methods and results. *J Dent Res.* 2005;84(2):118-32. doi: [10.1177/154405910508400204](https://doi.org/10.1177/154405910508400204).
- Fattah T, Kazemi H, Fekrazad R, Assadian H, Kalhori KA. Er,Cr:YSGG laser influence on microleakage of class V composite resin restorations. *Lasers Med Sci.* 2013;28(5):1257-62. doi: [10.1007/s10103-012-1200-6](https://doi.org/10.1007/s10103-012-1200-6).
- Hossain M, Nakamura Y, Yamada Y, Murakami Y, Matsumoto K. Microleakage of composite resin restoration in cavities prepared by Er,Cr:YSGG laser irradiation and etched bur cavities in primary teeth. *J Clin Pediatr Dent.* 2002;26(3):263-8. doi: [10.17796/jcpd.26.3.q8747j711g425582](https://doi.org/10.17796/jcpd.26.3.q8747j711g425582).
- Ertürk Avunduk AT, Bağlar S. Evaluation of microleakage in class v cavities prepared by different caries removal methods. *Microsc Res Tech.* 2019;82(9):1566-74. doi: [10.1002/jemt.23322](https://doi.org/10.1002/jemt.23322).