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Seeing is believing? When scanning electron microscopy (SEM) meets clinical dentistry: The replica technique.

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Abstract

In restorative dentistry, the in situ replication of intra-oral situations, is based on a non-invasive and non-destructive scanning electron microscopy (SEM) evaluation method. The technique is suitable for investigation restorative materials and dental hard- and soft-tissues, and its interfaces. Surface characteristics, integrity of interfaces (margins), or fracture analysis (chipping, cracks, etc.) with reliable resolution and under high magnification (from $\times 50$ to $\times 5,000$). Overall the current study aims to share detailed and reproducible information about the replica technique. Specific goals are: (a) to describe detailed each step involved in producing a replica of an intra-oral situation, (b) to validate an integrated workflow based on a rational sequence from visual examination, to macrophotography and SEM analysis using the replica technique; (c) to present three clinical cases documented using the technique. A compilation of three clinical situations/cases were analyzed here by means the replica technique showing a wide range of possibilities that can be reached and explored with the described technique. This guidance document will contribute to a more accurate use of the replica technique and help researchers and clinicians to understand and identify issues related to restorative procedures under high magnification.

KEYWORDS

clinical dentistry, magnification, replica technique, scanning electron microscopy, in vivo evaluation

1 | INTRODUCTION

Usually, in dentistry, the most used strategy for assessing the outcomes from clinical procedures is direct visual examination associated with auxiliary radiographic and other non-invasive image analysis (Azeem & Sureshbabu, 2018; Chen et al., 2017; Demarco et al., 2015; Demarco, Corrêa, Cenci, Moraes, & Opdam, 2012). Dental photography, by means of macro-lenses and, sometimes, in association with optical microscopy magnification ($\times 4$ to $\times 25$), has become also an important auxiliary method not only for diagnosis and treatment planning, but also

for short-, medium- and long-term follow-up investigations (Kalpana, Rao, Joseph, & Kurapati, 2018; Sitbon & Attathom, 2014).

Furthermore the use of optical microscopes in dental practice and a wide range of microscopy techniques have been used for dental research (De-Deus, Reis, & Paciornik, 2011; Hashimoto, 2010; Joshi, 2016; Schlafer & Meyer, 2015; Sharma et al., 2010). Particularly, scanning electron microscopy (SEM) has been applied to qualitative and quantitative investigations, with articles being published using SEM in dentistry since 1962 (Stewart & Boyde, 1962). An extensive list of possible applications can be used for analyzing dental materials,

biomaterials and oral tissues (Naves et al., 2011; Paradella & Bottino, 2012). Among all possibilities, an indirect detailed intra-oral examination, based on an epoxy polymer replica obtained by an accurate intra-oral negative imprint using an elastomeric material, has gaining notoriety in dental studies (Du, Zhao, & Swain, 2014; Koenig, Vanheusden, Le Goff, & Mainjot, 2013; Scherrer, Quinn, Quinn, & Wiskott, 2007; Yuce, Ulusoy, & Turk, 2019).

In restorative dentistry, the in situ replication of intra-oral situations, is based on a non-invasive and non-destructive SEM evaluation method, is suitable for investigation both sides: restorative materials and dental hard- and soft-tissues, and its interfaces. Surface characteristics, integrity of interfaces (margins), or fracture analysis (chipping, cracks, etc.) with reliable resolution and under high magnification (from $\times 50$ to $\times 5,000$). Making it a precise appliance for monitoring intra-oral processes over time, failure analysis of restorations and natural structures (fractography) [18] and routine evaluative comparisons. The SEM micrographs obtained from the replicas have also a very appealing didactic approach, besides its scientific purposes. However, to obtain a proper "replica" artefact-free with good quality to have trustable evaluation is material- and technique-sensitive (Scherrer et al., 2007). Recently, a detailed guidance (Scherrer et al., 2017) provided valuable information on how to obtain high-quality replica specimens for fractography analysis. However, a dedicated manual with a reproducible step-by-step, describing the replica technique for general applications, still lacking on dental literature.

Considering that minor details that leads to excellence and high fidelity in this kind of analysis, the aims of this report are: (a) to describe detailed each step involved in producing a replica of an intra-oral situation, (b) to exemplify an integrated workflow based on a rational sequence from visual examination, to macrophotography and SEM analysis using the replica technique; (c) to present three clinical cases documented using the technique.

2 | MATERIALS AND METHODS

Individual Protection Equipment (IPE) is mandatory (gaun, gloves, hats, and mouth masks), not only for safety reasons, but also to avoid any contamination of the specimens.

2.1 | Intra-oral impression procedures

The first step is to have an intra-oral replica from an in situ area is obtain a surface as clean as possible prior to impression procedures. The selected areas were cleaned with a lint-free blotting paper (or foam polymeric pellet) soaked in 70% (vol.) alcohol or NaOCl 2%, followed by water rinsing and then thoroughly air-dried. Any metal contact (tweezers, probes, etc.) were avoided on the surface. No pumice or powder particles were used to clean the area, as this will contaminate the elastomeric impression material and the final replica could be damaged with powder debris. A polyvinylsiloxane (PVS) one-step impression technique (Aquasil Ultra, Dentsply DeTrey, Konstanz,

Germany), using heavy and ultra-light consistencies, must be made for appropriate reproduction of the teeth and surrounding tissues. A polyether-based impression material is not advised because of chemical incompatibility with the epoxy pouring material (Scherrer et al., 2017). When the area of interest is a sub-gingival margin, and the extension of 0.5 mm into the gingival sulcus is required, a retraction cord (Cerkamed, Stalowa Wola, Poland) or a teflon cord 1/4 in. tape, USA Dental Tape, should be considered for better visualization. It is important to first make one negative imprint, only with the ultra-light consistency, which is expected to have adhered on it all debris, loose particles and any remaining biofilm from the area, being usually discarded. The second negative imprint is used for fabrication the replica, is also made with a fast set (2.5 min) ultra-light consistency and heavy body (Aquasil Ultra, Dentsply DeTrey) in a partial impression tray. After impression procedures, they must be ultrasonically cleaned in deionized water for 10 min, to remove all surface contaminants. Before pouring, the PVS impressions must be checked, under $\times 20$ to $\times 40$ magnification for any voids, irregularities and adhered particles. Negative imprints presenting any irregularity must be discarded and a new impression procedure needs to be made.

Note that not all the specimens needs to be cleaned, if the evaluating requires an "as it is" impression, for example, evaluation biofilm or calculus, the impression should be taken without any previous cleaning procedure from the area.

2.2 | Epoxy replicas

The cold mounting epoxy resin (EpoxyCure2, Buehler, Lake Bluff, Illinois) has a liquid-liquid formulation. Both liquids, resin and catalyst, present increased viscosity and its mixing requires careful manipulation, to avoid air entrapment and surface defects. Vacuum automatic mixer is highly recommended, although careful manual mixing is possible, which will be explained here in details. It is mandatory to use an inert glass recipe (e.g., glass becker) and inert mixing instrument (metal spatula). When mixing manually, carefully mix in "8-shaped" movements must be done without touching the glass becker and without removing the mixing instrument before finish the procedure. A completely and homogeneous mixture is achieved after 10 min of adequate manipulation. The glass becker needs to be cleaned immediately after the mixing procedure.

2.3 | Injection/pouring

To pour the negative imprint a syringe is used (#412, Covidien Monoject, Dublin, Ireland). Care must be taken not to include small air bubbles and the tip of the syringe needs to stay in contact with the impression material surface while dispensing it. The negative imprints are poured with cold mounting epoxy resin with a minimum height of 20 mm, additional walls may be needed. This ensures efficiency, ease of removability from the mold, and reduces risk to undesired fracture and also allows to use less material. To avoid internal bubbles or

porosities at the epoxy model surface, the mold with uncured epoxy was cured in a cold-dry pressure pot, under two bars, during 24 hr.

2.4 | Specimens dimension adjustment

After the replicas are removed from their PVS molds, another meticulous checking procedure has to be performed at this stage, under $\times 20$ to $\times 40$ magnification, for any positive or negative irregularities at the epoxy model. Replicas presenting any irregularity must be discarded and a new pouring procedures needs to be done. After the separation from the PVS molds, the area to be evaluated has to be cut to the adequate dimensions and orientation plane (IMAGE). After cutting procedures, a ultrasonically cleaning in

deionized water for 10 min, is mandatory to remove contamination from the specimen's surface.

2.5 | Scanning electron microscopy evaluation

The reduced epoxy replicas were fixed on aluminum stubs using double face carbon tape (8 mm, EMS, Hatfield, PA) and then gold sputtered (one cycle of 180 s) under a vacuum atmosphere, in a sputtering device (MED 010, Balzers Union, Balzers, Liechtenstein). For the selected replicas, surfaces were examined by SEM (LYRA SEM-FIB, Tescan, Brno, Czech Republic), focusing, in a first moment, on integrity and signs of and non-homogeneous surface, that is, looking for gross alterations along the replica surfaces. After these first checking procedures, the

FIGURE 1 Case 1: (a.1) SEM micrograph initial situation, an unsatisfactory composite restoration, with a bulk fracture and open contact point ($\times 38$). The squared area delimited by pointed line refers to higher magnification area shown in a.3; (a.2) intra-oral picture from just before preparation procedures; (a.3) close-up at mesial aspect of the restoration, showing a fracture line (arrows) and an open contact point (pointer) ($\times 88$); (b.1) SEM micrograph from the cavity after the initial preparation; (b.2) intra-oral picture from the same treatment stage; (b.3) SEM micrograph from the same stage, but with different inclination ($\times 42$); (c.1) SEM micrograph from the cavity after IDS procedures ($\times 37$). The squared area delimited by pointed line refers to higher magnification area shown in c.3; (c.2) UV-filtered intra-oral picture after IDS procedures, the arrow depict the IDS layer, it is possible to note higher UV reflectance; (c.3) SEM micrograph from the same stage, however, with under higher magnification ($\times 794$); (d.1) SEM micrograph from fitting evaluation of the final ceramic restoration ($\times 37$). The squared area delimited by pointed line refers to higher magnification area shown in d.3; (d.2) picture from the final restoration fitting on the cast model; (d.3) SEM micrograph showing intra-oral fitting test (pre-bonding stage); (e.1) SEM micrograph showing the restoration after bonding, finishing and polishing procedure ($\times 37$). The squared area delimited by pointed line refers to higher magnification area shown in e.3; (e.2) intra-oral picture; (e.3) SEM micrograph showing the same area d.3, however, after-bonding stage ($\times 794$). To: tooth structure; Re: restoration; Co: composite; De: dentin; En: enamel; Ce: ceramic; ECe: etched ceramic [Color figure can be viewed at wileyonlinelibrary.com]

macrophotography could be used as a guide to obtain correspondent images. The specimens were examined under magnification varying $\times 50$ to $\times 4,000$. The unit operated at 20 kV, WD range of 5–15 mm, and with a spotsize range of 25–100 pA.

3 | RESULTS

Three clinical situations/cases were analyzed here by means the replica technique. The aim from this chapter is not discuss outcomes or operative techniques, however, the main goal is to show a wide range of possibilities that can be reached and explored with the replica technique.

Case 1: Female patient, 32 years, presenting a MO, non-satisfactory composite restoration, complaining about absence of contact point, at the mesial site of tooth 16 (a.1). After, initial intra-oral examination, small marginal irregularities were detected and an initial photographic protocol was done. In between every operative step, a negative imprint was made following the protocol as described in Section 2. Five main steps from the procedure are shown here (not all operative steps were included in the analysis), and intra-oral macrophotography were used in a associative manner with micrographs. At the image a.2, the use of an UV-filter (fluor_eyes, Emulation, Frankfurt, Germany) aiming to differentiate the dental hard-tissues, from the adhesive resin composite layer (IDS layer), in the image c.3, it is even possible to differentiate precisely dentin, enamel and the adhesive (IDS) layer. Another possible utilization from the technique is the comparison of the same area after different successive steps: d.3—pre-bonding stage (or the dry fitting test of the restoration) and e.3—after bonding stage, showing the external marginal interface (Figure 1).

Case 2: Female patient, 52 years, patient complaining about color and shape of central incisor (11). The SEM sequence here were used to show and evaluate isolation retraction procedures using teflon tape

at the gingival sulcus and also correlate it with well-polished surface, due easy access as a result from proper retraction (a and d). The SEM images also show a smooth transition between the ceramic veneer and the tooth surface, after the polishing procedures no excess from the bonding material was detected which has influence to soft-tissue integration to restoration (b and e), presenting a healthy aspect of the soft tissues (f). For this specific analysis, the impression procedures need a different approach. When clamps, metallic matrices, and other restorative-aid devices are in place, those impressions should be done avoiding all undercuts and minimizing the amount of impression material used, applying it only at the area of interest for the analysis. Blocking undercuts with teflon tape and careful attention to how and to where the material flows are mandatory (Figure 2).

Case 3: Female patient, 49 years, 9 years follow-up of ceramic veneers. The retraction cord (pointer in b) was used to better assessment of the cervical area. It is possible to identify some marginal gaps and some roughness on the ceramic surface (b), probably due to inappropriate resin cement removal using rotatory instruments or due abrasion of the resin cement caused by toothbrush over the years. An area of adhesive continuity (AAC) (de Andrade et al., 2013) can be seen at the SEM micrograph (c). The AAC is defined as hybrid interface of different structures that have been bonded together, in this case, enamel, bonding agent, resin cement, and a dental ceramic (IPS D'sign-Ivoclar Vivadent) (Figure 3).

4 | DISCUSSION

Science improves in small steps, this also counts for dentistry, there is still plenty of room at the bottom (Feynman, 1992), and to understand better at a microscopic scale what clinicians in dentistry are doing in everyday clinical practice is key. SEM has been used in dental science for a long time and diverse number of analysis and investigations,

FIGURE 2 Case 2: (a) Macro-photography showing the prepared tooth and the isolation at the cervical area. (d) SEM micrograph from the same area showing details from the retracted tissue; (b) macro-photography showing the ceramic veneer immediate after bonding, finishing and polishing procedures; (e) SEM micrograph showing adhesive continuity area with a smooth transition from ceramic surface to tooth cervical surface; (c) although immediately after bonding, finishing and polishing procedures the soft-tissue shows a minor damage, after 14 days (e) the restoration shows optimal integration with the surrounding tissues (pointer on a, b, and e—pointing teflon tape, an auxiliary tissue retractor with non-adhesive properties that helps in excess material removal after bonding). Ce:ceramic; To: tooth surface [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 3 Case 3: (a) Macro-photography showing 9 years follow-up of ceramic veneers; (b) the retraction cord (pointer) was used to better assessment of the cervical area, some marginal gaps were detected and also roughness on the ceramic surface; (c) an area of adhesive continuity can be seen at the SEM micrograph. Ce:ceramic; To: tooth surface; Co: composite [Color figure can be viewed at wileyonlinelibrary.com]

most of it restricted to in vitro and ex vivo analysis (Paradella & Bottino, 2012). A range of magnification scales has on one end the naked eye view, passing by lens magnifications, macro photography side-chair optical light-microscopy, laboratory optical light-microscopy and at the other end the replica technique using SEM. With SEM it is possible close the gap between clinical requirements and scientific needs (Scherrer et al., 2007). Approaching the oral cavity from an “in-vivo like” perspective, under high-magnification ranges, by means of a non-destructive and non-invasive analysis, opens a new and wide universe of possibilities. Besides, with the increase in the use of adhesive procedures and consequently the need to understand the interface dynamics, established after performing a restoration bonded to the dental structure, new ways of investigating these variations become indispensable in dentistry. An example in how is important this understanding, is the adhesion of indirect restorations, where there are a variety of critical factors, which influence longevity. The use of this method based on replica and SEM analysis makes more precise, the characterization of the different types of interface, present in modern dentistry, due to the great variety of materials and techniques.

This technique enables indirect evaluation of almost any kind of intra-oral surfaces: external interfacial zones, surface of restorative materials, natural morphology from hard and soft tissues, comparison overtime, aging management, failures enabling fractography analysis of failures, cracks, chipped surfaces, among other possibilities (Scherrer et al., 2007). However, to obtain artifact-free micrographs it is mandatory to carefully follow the steps and secure manipulation of the materials as clean as possible. The final outcome, that is, the SEM micrograph will never have higher quality than the quality from each step taken during the confection of the specimens. The final SEM

micrograph is only an image registration, under high magnification, of the whole process.

In this context, all the steps described in this text must be conducted not only with all operative skills from a dentist, but also must be faced as a forensic approach. Obtaining a negative imprint here, for example, is more related to collecting evidence than a simple clinical step. It means that the collection, preservation, processing and analysis of the evidence are often critical in determining the quality and reliability of the final SEM image (Paradella & Bottino, 2012). The intra-oral site of a fractured restoration, for example, should be addressed with as much as attention to do not alter the “crime scene”.

Within this point of view, it is key to understand that the SEM micrographs will always give a better contribution when in association with other information, or, other evidences. The replicas models can be considered a *physical evidence*, also called real evidence: actual tangible evidence that can be touched and picked up, among other examples includes fractured parts of a restoration and/or tooth, cast models; the SEM micrographs images are *demonstrative evidence*: requires a demonstration or an explanation by an expert in the field: an experienced microscopist with familiarity with the microscopic aspects of oral tissues and biomaterials. Intra-oral, macro photography, auxiliary radiologic examinations (x-rays, MRI's, CT scans), also figures in the same category; *Testimonial evidence*: brought forth by witnesses, such as testimony, in the case, the patient's declaration on what happened when he or she experienced the failure; *Documentary evidence*: these evidences are documents like patient's register data at the dental office, treatment plannings, previous photographic and auxiliary examinations, referral letters, etc. All the “evidences” set will help to solve a puzzle, after piecing them together (Soule, Svensson, & Wendel, 1966).

Furthermore, for correct and efficient interpretation of the micrographs obtained by SEM analysis, the microscopist must be able to discriminate normal, non-normal microscopic aspects and technique artifacts. Therefore, training and a certain level of experience in surface analysis and characterization of dental materials and oral tissues is mandatory. When analyzing *ex vivo* specimens, extracted teeth, fractured parts from a clinical situation, the composition analysis is possible. When evaluating replicas, composition analysis like energy-dispersive spectroscopy (EDS) (Paradella & Bottino, 2012), is not possible. Thus, it is a crucial need to have a good level of electronic microscopy skills to identify and differentiate the nature and composition of what it is being analyzed. Furthermore, artefact identification could be even harder when dealing with replicas, due limits of precise detail's reproduction of the impression material and copy fidelity from epoxy resin.

Despite technical sensitivity, we conclude that the scanning electron microscopy could be considered a reference standard for high magnification clinical evaluation, and has a potential for follow-up studies by means of specific comparisons over the time.

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