Using an SEM as an ESEM to Study Minute Human Bloodstains on Stainless Steel

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BIOGRAPHY

Dr Policarp Hortolà graduated in biological sciences and then did a doctoral programme on the sedimentary record and palaeoenvironmental evolution at the University of Barcelona. He received his PhD degree from the Rovira i Virgili University for a thesis on the morphology of mammalian erythrocytes in bloodstains, which had a prehistorical bias. He is currently working as a senior researcher and lecturer at Rovira i Virgili University.

ABSTRACT

Because many conventional, high-vacuum scanning electron microscopes (SEMs) are still in use, their full potential should always be explored. With this aim in mind, two uncoated stainless-steel blood lancets, used for finger puncturing in a study carried out 20 months earlier, were examined for possible blood smears in an SEM using secondary electron imaging at an accelerating voltage of 0.5 kV. Minute bloodstains and some of their erythrocytes were found. As was previously revealed in uncoated bloodstains on stone, this study clearly demonstrated that a conventional SEM can be used in secondary-electron mode just like an environmental SEM to examine these biological materials on a stainless-steel substrate.

KEYWORDS

scanning electron microscopy, environmental scanning electron microscopy, blood smears, red blood cells, haemotaphonomy, specimen preparation

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INTRODUCTION

The presence of erythrocytes (red blood cells, RBCs) in a smear is evidence of blood [1]. RBCs consist of a composite membrane surrounding a fluid interior. This composite membrane is composed of a plasma membrane plus a membrane skeleton. Throughout the zoological class Mammalia, RBCs are anucleate, which means that under physiological conditions mammalian erythrocytes are typically shaped as biconcave discs (discocytes). In the Camelidae family, however, RBCs tend to be flatter, and oval (ovalocytes). Other physiological shapes, which are either minor or pathological, are echinocytes (burr cells), codocytes (target cells), keratocytes (horn cells), torocytes (doughnut cells), drepanocytes (sickle cells) and many other morphologies [2, 3].

The morphological preservation of mammalian RBCs in bloodstains has been reported even in palaeolithic tools estimated to date back as far as 2 million years [4]. A smear may be defined as the result of a causal relationship between a physical contact (the cause) and a trace (the effect). This causality is implicit in Locard's Principle of Exchange: "Every contact leaves traces," and agrees with the presence of blood residues on both ancient (archaeological) and modern (court-evidence) implements. Apart from this deterministic principle, in a broad sense, all experimental frameworks are based upon Lyell's geological Principle of Actualism, usually summarised as: "The present is the key to the past."

One instrument that can be used successfully for the microstructural characterisation of erythrocytes in blood smears is the scanning electron microscope (SEM). The cytomorphological SEM approach to bloodstains (haemotaphonomy) has been developing for the last fifteen years [5]. Moreover, the fact that SEMs can examine objects at very low magnifications means that they are very useful tools in both forensic and archaeological studies [6]. The SEM technique is suitable for use in bloodstains concomitantly with a previous in-situ screening of the smears (i.e. with an orientative test to make a generic diagnosis of bloodstains as such). This screening consists of observing: (a) the stain's shape (e.g. a projected staining shape in small smears), appearance (pasty, dense look) and dark red colour (if the substrate is paler than the smear); and (b) the positive reaction to both the blood (haemoglobin/myoglobin) and the protein (albumin) fields when compared with a compensation area (blank) in colorimetric test strips for rapid determination in urine [7].

The emergence of variable pressure SEMs (often referred to as environmental SEMs) was an important advance in electron microscopy because their working mode allows the examination of samples without coating. It has been widely assumed that insulating specimens are subject to charging and, therefore, image formation is unsatisfactory. The group of non-conducting specimens contain such inorganic and organic materials as minerals, rocks, and biological specimens in which the water has been removed by drying, substituted with low-vapour-pressure polymers or frozen *in situ*. In order to overcome charging problems, a typical procedure for conventional SEM involves depositing a 1-10 nm thick conductive coating of carbon or metal (e.g. gold, gold:palladium 4:1 alloy, platinum, tantalum,or iridium) on the samples. This is generally done by vacuum evaporation or sputtering. In the evaporation method, the coating material is heated to its vaporization temperature in a high vacuum and the evaporated coating atoms land on the specimen surface. In

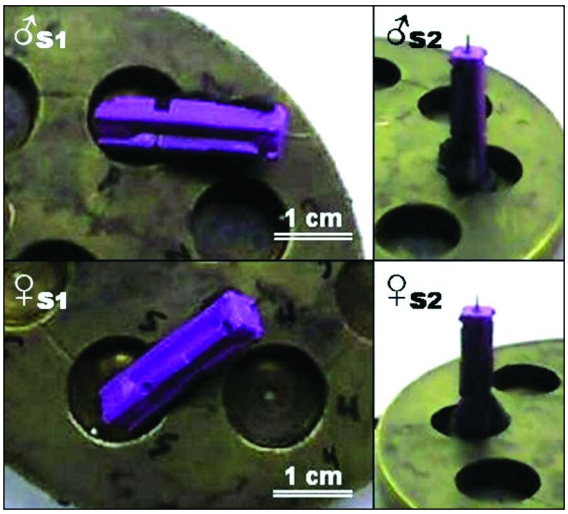

Figure 1:
Photographs of the uncoated lancets after fixing to the SEM sample holder. The full SEM examination took place in two sessions on different days, both samples being examined during each session.
Top, left and right: The lancet used to puncture the male's fingertip.
Bottom, left and right: The lancet used to puncture the female's fingertip.
S1 = session 1; S2 = session 2.

the sputtering method, a negatively charged coating material is bombarded by positive ions in a low-vacuum gas discharge and the eroded coating atoms land on the specimen surface [6]. Although some coatings have been successfully removed from the samples without morphological damage (e.g. gold from animal exoskeletons [8], or silver from microfossil shells [9]), one disadvantage of coating materials is that they are often difficult to remove. Furthermore, the coating process may damage delicate specimens, such as heat-sensitive biological tissues. These considerations are of particular importance in archaeological or forensic samples that are to be be exhibited in museums or in court.

Because many (less-advanced) high-vacuum, conventional SEMs are still in use, it makes sense to determine whether they can be used as environmental-like SEMs. In a previous paper, I reported the use of a conventional SEM to examine human RBCs in bloodstains and their (stone) substrates at magnifications up to 10,000×, without any specimen coating [10]. Those bloodstains were obtained using stainless-steel clinical lancets. In this article, I report the direct examination of those lancets without any previous specimen preparation, using the same conventional SEM.

MATERIALS AND METHODS

The tips of the righthand forefingers of an adult male and of an adult female were punctured with a FinePoint sterile stainless-steel, plastic-handled clinical lancet (LifeScan, Inc., Milpitas, CA, USA). The lancets were then left indoors, out of direct sunlight and air currents, for about 1.5 hours, and stored in a non-hermetically sealed plastic box. After 20 months, the lancets were fixed to an SEM sample holder using removable Leit-C-Plast plastic conductive carbon cement (Bal-Tec, Münster, Germany), as shown in Figure 1. No biological specimen preparation (i.e. fixation, conducting infiltration, dehydration, embedding or coating) was used. The uncoated samples were then directly examined for possible blood smears, using secondary electrons, in a JEOL JSM-6400 scanning electron microscope. The specimens were examined together in two sessions on different days. Except for the working distance (WD), the remaining working conditions were those previously standardised for uncoated, untreated bloodstains on stone: an accelerating voltage of 0.5 kV, a probe current of 3×10^{-11} A, and an image-recording resolution of 1024×832 pixels [10]. The WD varied from 10 to 17 mm. Micrographs were recorded as digital images with an INCAEnergy system (Oxford Instruments NanoAnalysis Ltd, UK).

RESULTS

The two lancets showed minute bloodstains on their surface. Given their identity, the mechanism most likely to have produced the smears was internal contact with the blood vessels of the fingers. Although the smears were mainly seen as blood plasma in the process of detaching from the steel substrate, several minute bloodstains displayed RBCs on their surface. Low magnifications resulted in better resolution. At high magnifications, the resolution of RBCs on a stainless-steel substrate was a little lower than that of previously examined erythrocytes on non-conducting rock substrates (limestone and chert). Some representative results are shown in Figures 2 to 6.

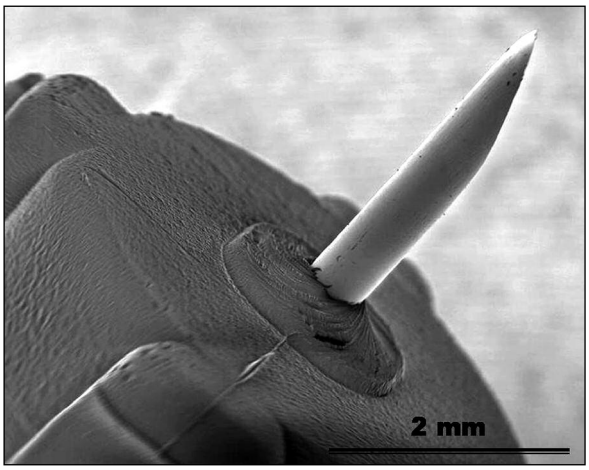

Figure 2:
Lancet with the male's bloodstains at very low magnification.
Original SEM magnification = 20X.
Working distance = 14 mm.

Figures 2 and 3 display, at very low magnification, the bare (i.e. uncoated) stainless-steel lancets with the male's and female's bloodstains. In Figure 4, some scattered blood smears on the head of the lancet used on the male are shown at low magnification. Figure 5 shows, at low magnification, several bloodstains and steel surface irregularities filled with blood, close to one of the flattened faces of the head of the lancet used on the female. In Figure 6, some RBCs can be seen on the heads of the two lancets at medium and high magnifications. In the lancet with the male's bloodstains, at medium magnification the erythrocytes can be seen one after the other as in a 'rouleau' (Figure 6a). At high magnification, plasma-free RBCs can be seen lying flat on the surface of the lancet with the female's bloodstains (Figure 6b). The distorted appearance of these erythrocytes is not an imaging artifact but probably due to the bloodsmearing mechanism.

DISCUSSION

The bloodstains on the stainless-steel lancets were preserved even though it is difficult for a clinical lancet to be sufficiently smeared with blood at the time of puncturing. This difficulty arises because of the speed of the puncturing action. Moreover, even if they are sufficiently smeared, one further difficulty is to retain the blood smears for 20 months without them detaching from the (low-adhesive) steel surface. From a theoretical point of view, short-term preservation of specimens is a *sine qua non* precondition for longer-term preservation. This preservation of bloodstains can be valuable, particularly with untreated samples, because they can be directly dated, for example by electron spin resonance [11].

On the whole, there do not seem to be big differences between the secondary-electron images of uncoated bloodstains on stainless steel or stone, except for a slightly better image resolution for RBCs at high magnifications when the bloodstains are on stone. In general, the lower RBC image resolution at high magnifications is related to the low voltage used in the uncoated sample examination. Accelerating voltages higher than the 0.5 kV used in both this study and the preceding one would give higher resolutions, but at the same time they might damage the samples. On the other hand, the lower resolution in the lancets could also be due to the lack of homogeneity in the electron conductivity between the (insulating) blood smears and their (conductive) stainless-steel substrate. This resolution was not so low when the non-conducting smears lay on a similar insulating substrate such as rock [10]. And although in the present study it

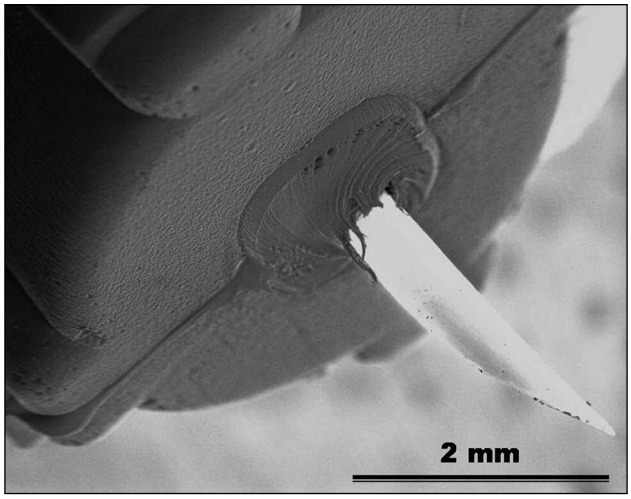

Figure 3:
Lancet with the female's bloodstains at very low magnification.
Original SEM magnification = 20X.
Working distance = 12 mm.

was low, the resolution did not inhibit RBC visualisation in the lancets at all, so the diagnostic value of the SEM images remained intact. Furthermore, the distorted appearance of some of the RBCs examined was probably due to the blood smearing mechanism, not an imaging artifact. As in the previous work on uncoated blood smears on stone, magnifications over 10,000× were not attempted because higher magnifications are not required to clearly visualize the erythrocytes of blood from any mammalian species [12].

From the haemotaphonomical point of view, the types of substrate (stone, metal, paper, etc.) do not seem to play a major role in the morphology of erythrocytes, as long as they have similar haemotaphonomically-significant physical properties (mainly topography, roughness, texture, permeability, absorbency and fissuration) [13]. The RBC morphology is encoded in the mechanical properties of its composite membrane (i.e. the plasma membrane plus the membrane skeleton). The plasma membrane provides bending rigidity, and the membrane skeleton stretch and shear elasticity [14]. While the plasma membrane is the lipid bilayer that contains proteins such as ankyrin and band 3, the membrane skeleton is a skeletal network of proteins, mainly spectrin, actin, and band 4.1. This membrane skeleton is localized exclusively on the cytoplasmic surface of the plasma membrane [15]. The fact that the red cells are preserved intact in bloodstains can probably be explained by the same membrane mechanical properties as under physiological conditions.

CONCLUSIONS

As was previously found in blood smears on stone, this study also reveals that a conventional SEM can be used in secondary-electron mode just like an environmental SEM to examine bloodstains on a stainless-steel substrate. This concise method using uncoated specimens clearly questions the need for sample coating in conventional SEMs as it provides usable images with a substantial saving in terms of the condition of the sample, time and money. While this is mainly a scientific and technical issue of special relevance to forensic science and archaeology, it also involves the ethical consideration of the life span of scientific equipment in (and co-operation with) the developing countries.

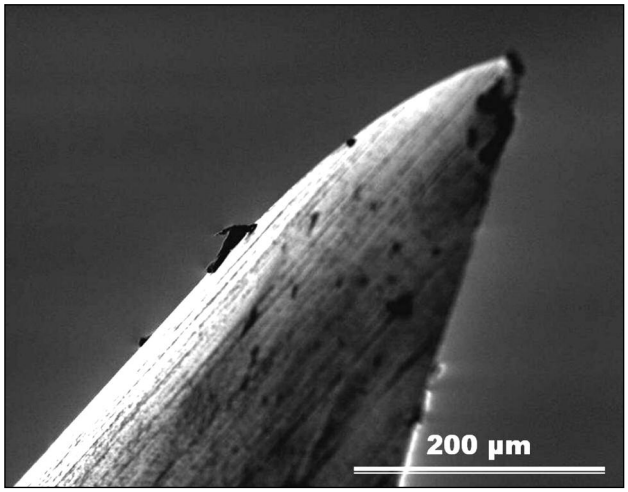

Figure 4:
Lancet head with the male's bloodstains at low magnification. Some scattered blood smears can be seen on the bare stainless steel.
Original SEM magnification = 200X.
Working distance = 13 mm.

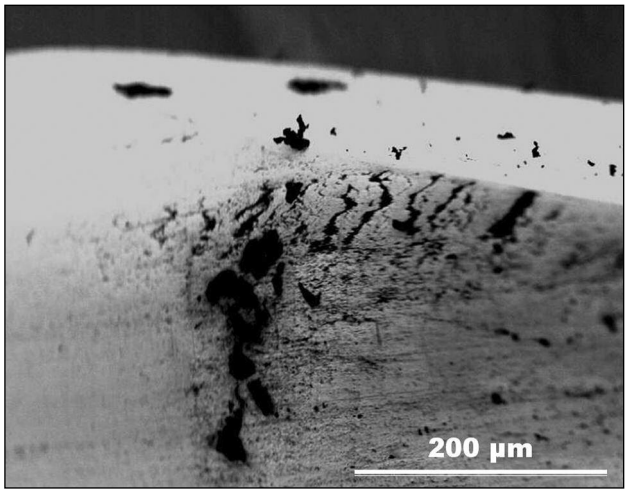

Figure 5:
Lancet head with the female's bloodstains at low magnification. Steel surface irregularities filled with blood and blood smears can be seen on the bare stainless steel close to one of the lancet's flattened faces.
Original SEM magnification = 200X.
Working distance = 13 mm.

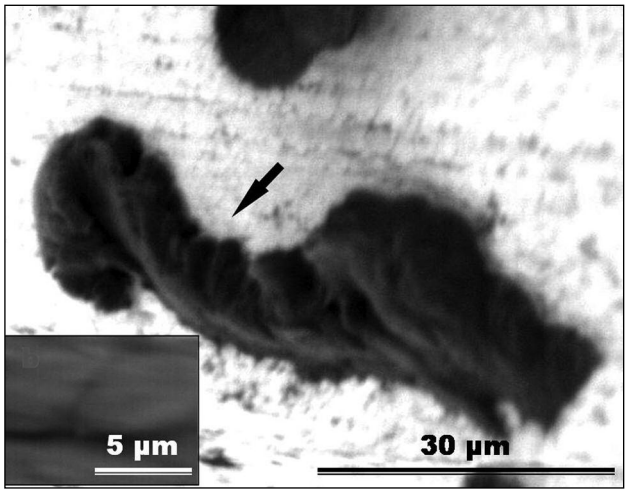

Figure 6:
Erythrocytes as seen at medium and high magnifications.
Blood on the lancet head with the male's bloodstains, close to one of its cutting edges. The RBCs appear one after the other, as in a 'rouleau'. Original SEM magnification = 1,500X; WD = 15 mm.
Inset: RBCs on one of the flattened faces of the lancet head with the female's bloodstains. The fact that the image resolution is low is related to the low voltage used in the SEM, but it could also be due to the lack of homogeneity in the electron conductivity between the bloodstain and the stainless-steel substrate. Original SEM magnification = 10,000X; WD = 11 mm.

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