# Comparison of Advanced Sample Preparation Techniques for High-Resolution Imaging of Sponge Spicule Cross-Sections

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**Abstract** This study comprehensively investigates the effectiveness of three advanced sample preparation techniques—ion milling, laser cutting, and Focused Ion Beam (FIB) milling—for high-resolution imaging of Euplectella aspergillum sponge fibers called spicules. Primarily composed of silica layers with nanometer-scale organic interlayers, spicules possess complex hierarchical structures which are critical for their mechanical properties. Accurate characterization of these structures requires advanced sample preparation to prevent artefacts and preserve structural integrity. Ion milling introduced significant surface degradation and uneven material removal in spicules. Despite its precision, laser cutting caused thermal damage and induced micro-cracks, compromising the microstructural integrity. In contrast, FIB milling provided superior results, producing smooth, artefact-free cross-sections with minimal thermal and mechanical stress. The real-time imaging capability of FIB milling further ensured optimal sample preparation, making it the most suitable technique for delicate biological materials like spicules. The findings of this study provide valuable insights into the preparation of biological samples for further research and analysis.

**Keywords** Spicule characterization · Nanoscale imaging · Laser-based techniques

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## Introduction

The spicules of the Venus Flower Basket (Euplectella aspergillum), a deep-sea glass sponge, are known for their remarkable structural design and strength. Made mainly of silica, these spicules have a layered structure, with a central organic core surrounded by concentric rings of silica that vary in thickness [1]. This unique design gives the sponge both strength and flexibility, allowing it to withstand the high pressures of the deep ocean without breaking [2]. The spicules are arranged in a cylindrical lattice, which makes the sponge's body stable and strong, helping it stay anchored to the ocean floor. Understanding the detailed architecture of spicules is essential not only for unravelling the biology and ecology of sponges but also for exploring the potential applications of this natural architecture in the development of advanced structures. The nanoscale features and microstructure of spicules are key factors in their exceptional mechanical properties. This makes them a valuable subject of research for developing new materials that mimic these natural designs [3–5].

The characterization of spicules involves high-resolution imaging techniques capable of revealing their internal structures in great detail. Techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) are commonly used to examine the morphology, composition, and mechanical properties of spicules [6, 7]. However, the success of these imaging techniques largely depends on the quality of the sample preparation process. Proper preparation is essential to reveal the internal structure of spicules without causing any damage, which could affect the accuracy of the analysis. Given the delicate nature of spicules and their susceptibility to thermal and mechanical stresses, sample preparation methods must be carefully chosen and executed to preserve the fine structural details [8].

Laser cutting technique works by using a focused laser beam to cut through materials by heating them along a specific path. This technique is effective because it allows samples to be cut quickly and accurately with minimal physical contact, reducing the risk of mechanical damage [9]. Huang et al. demonstrated the effectiveness of laser cutting in fabrication hallow fibers, showing that it is particularly useful for processing different materials that are difficult to handle with traditional methods [10].

Ion milling is another important technique to cut the samples in materials science, especially for preparing samples for transmission electron microscopy (TEM) [11, 12]. Ion milling relies on sputtering, in which energized ions physically eject other atoms and molecules from the sample surface through momentum transfer. Ion milling is commonly used in materials science and engineering for applications such as cross-sectioning samples, thinning samples for electron transparency, and removing layers for deeper analysis. It creates smooth, polished surfaces, which are perfect for high-resolution imaging [13, 14].

Focused Ion Beam (FIB) milling is another highly precise technique used to prepare samples at the nanometer scale. FIB uses a finely focused beam of ions, usually, to carefully remove material, allowing for extremely fine and accurate cuts.

This method is especially valuable for handling delicate samples, such as biological tissues and microelectronic components. FIB milling also offers the advantage of in situ imaging during the milling process, which helps ensure the quality and precision of the final cross-sections [15].

There are two main methods for sample preparation using ions: focused ion beam (FIB) and ion milling (also known as near-parallel ion technique). The primary difference between these methods is in the ion beam characteristics. FIB uses a highly focused beam of high-energy ions, typically gallium, at energies up to 30 keV, allowing for precise milling in very small areas (around 50 × 50 μm). However, this process can be time-consuming due to its precision. On the other hand, ion milling uses lower energy ions, usually less than 20 keV, and creates a broad, unfocused ion beam from an inert gas like argon. This beam is directed at the sample surface, where the ion's kinetic energy is converted to heat and momentum, causing atoms to be detached and gradually removing material in thin layers. By moving the ion beam across the sample, the surface can be milled to the desired depth with control over the milling rate, resolution, and surface quality. Over time, advancements in ion sources, beam optics, and sample handling have significantly improved the precision and efficiency of these techniques [16].

In this study, we systematically evaluate how well ion milling, laser cutting, and FIB milling work for preparing cross-sections of spicules for high-resolution imaging. By comparing the results of these methods, we aim to find the best technique for preserving the structure of spicules during sample preparation. This research not only advances our understanding of sponge biology by improving how we study spicules but also has wider applications for preparing other delicate biological specimens in materials science and biomimetics.

## Materials and Methods

Three groups of spicule bundles were carefully extracted from the sponge skeleton using a Dremel. The first set of bundles was securely affixed to the laser cutting platform using a tape to prevent any movement during the cutting process. The Universal M-360 Laser Cutter was used for this procedure. The laser power was adjusted to 5% of the machine's maximum capacity, while the cutting speed was maintained at 2% of the machine's maximum speed. The process was controlled using EngraveLab v10 software. The next collection of spicule bundle extracted was attached to the stage of the Ion Beam Milling System (Leica EM TIC 3X) using carbon tape. The ion energy was set to 6 keV, and a beam current of 2.2 mA was chosen based on the manufacturer's guidelines. To provide mechanical support during the FIB milling process, the third group of bundles of spicules were affixed to a stub using hot glue. The sample was placed in a Scanning Electron Microscope (SEM, Thermo Scientific™ Scios), and the FIB system was operated at a low beam current 65 nA and voltage (30 kV) to achieve precise material removal while minimizing surface damage.

## Results and Discussion

In laser cutting, the laser's energy caused rapid, localized heating, which could lead to melting, vaporizing, or even destroying parts of the spicules, as shown in Fig. 1. This heat could damage the spicules, causing them to crack, warp, or melt. Moreover, the quick heating and cooling during laser cutting could create mechanical stress in the spicules, leading to tiny cracks. These cracks made it difficult to get a clear, detailed view of the spicules, which was important for studying their structure.

Figure 2 shows the SEM images of the cross-sections of spicule bundles after cutting by the ion milling method. The highlighted areas show noticeable surface damage, including rough textures. The spicules were deformed and partially melted due to the localized rise in temperature during the process, especially in the circular cross-sections where ion milling caused significant distortion. These issues made it difficult to see the fine details of the spicules, affecting the quality of the analysis. The main problem with ion milling for spicules was that the high-energy ions can damage the surface. This damage could result in rough surfaces and defects that change the spicule's natural structure. Although this method was not suitable for cutting spicule cross-sections, other studies have demonstrated its effectiveness with different materials.

Wei et al. [17] used ion milling to create a smooth surface for easier observation and to minimize the impact of artificial fractures. The three-beam ion polishing

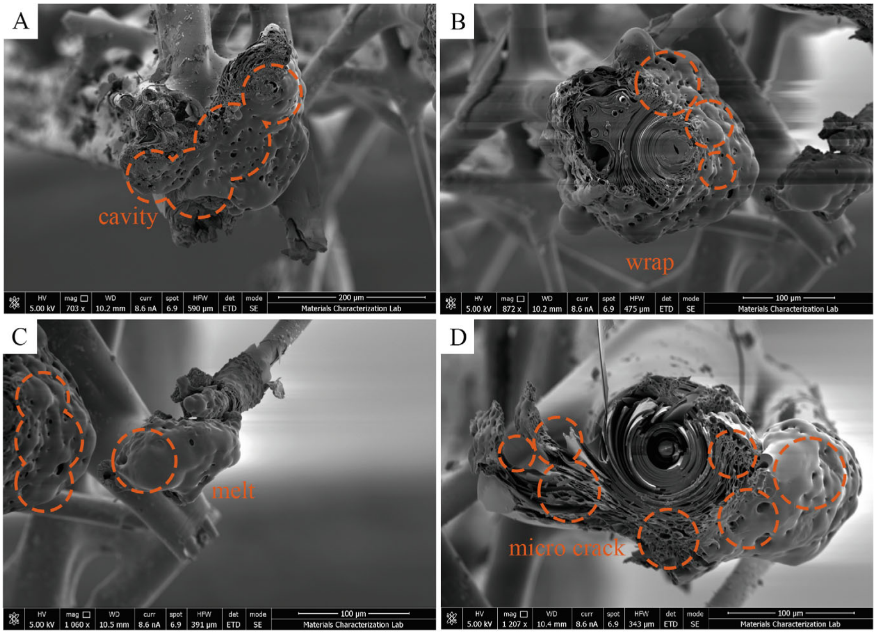


**Fig. 1** SEM images **a–d** of spicule bundles after cutting the samples by laser cutting technique

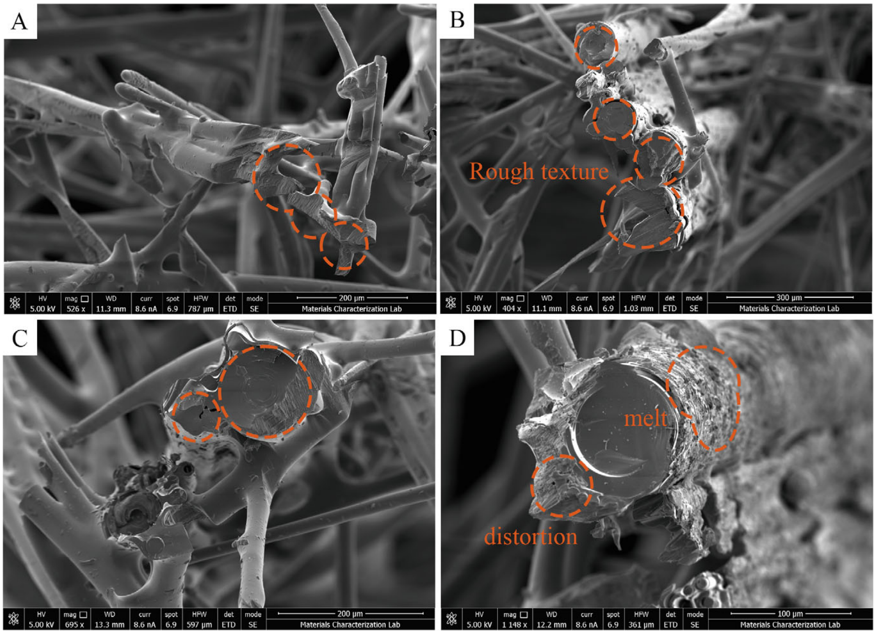


**Fig. 2** SEM images **a**–**d** of spicule bundles damaged after being exposed to ion milling

method removed approximately 8 μm of surface material, resulting in a flat, rectangular organic-rich shale surface ideal for scanning electron microscope (SEM) observation. Coutinho et al. [18] utilized ion milling for the preparation of tooth-biomaterial interfaces for transmission electron microscopy (TEM) analysis. While the TEM successfully revealed the structural details of the interface, the high-energy ion milling process introduced molten areas and fogging on the cross-section of the sample. Kleiner et al. [14] demonstrate that argon broad ion beam (BIB) sectioning is an effective method for preparing high-quality surfaces of hydrated alite for scanning electron microscopy (SEM) imaging. It produced flat, smooth surfaces with minimal artifacts, allowing for high-resolution imaging of nano-sized pores. However, in our study, the ion milling technique resulted in significant damage to the samples and proved to be an unsuitable method for sectioning the spicules.

In addition to the above-mentioned techniques, FIB milling was used to prepare spicule cross-sections. Figure 3 shows the SEM images of the cross-section of spicules milled using FIB. The surfaces created by FIB milling were smooth and didn't have the damage seen with other techniques. This showed that FIB method is a better solution to cut the delicate biological samples such as spicules. One of the main advantages of the FIB milling technique is the lower temperature generated during the milling process compared to the other techniques. The gentle sputtering action of the ion beam also reduced mechanical stress, preventing the formation of tiny cracks or other structural damage. This makes FIB milling an excellent choice for preparing spicule cross-sections as it preserves the fine details of the structure

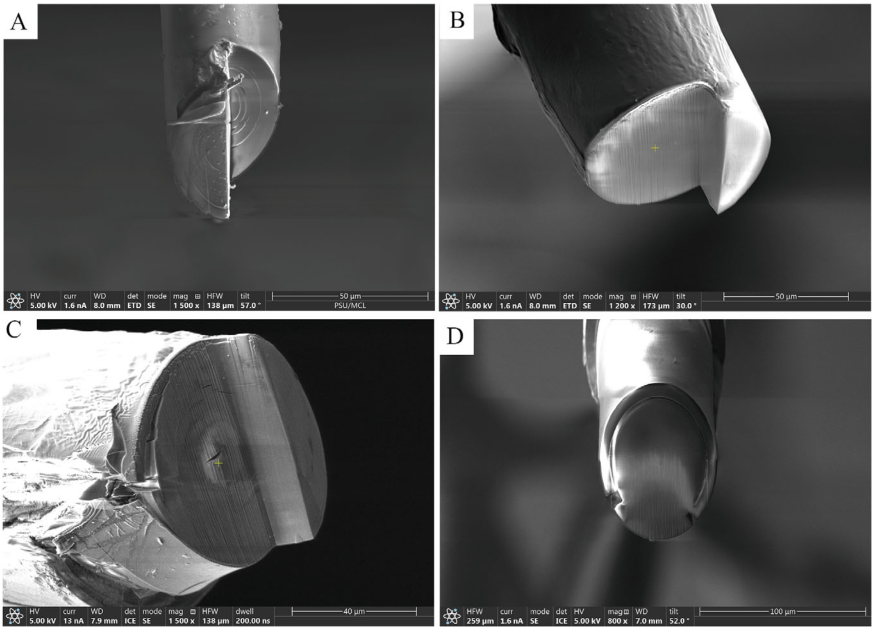


**Fig. 3** SEM images **a–d** of spicule cross-sections cut with FIB milling. The surfaces are smooth and free of damage or heat effects, showing FIB's precision in maintaining spicule integrity

and avoids introducing any unwanted changes. Additionally, FIB systems allow for real-time imaging during the milling process. This means researchers can adjust the process immediately, ensuring that the cross-sections meet the required standards and are free from defects.

Our study using focused ion beam (FIB) techniques aligns with the findings of Giannuzzi et al. [19]. They successfully employed FIB to prepare multiple scanning electron microscopy (SEM) images from sequential cross-sections. This approach allowed for both two-dimensional and three-dimensional analyses of bone/dental implant interfaces. In contrast, our results differ from those of Meerbeek et al. [20]. Their application of FIB to the resin-dentin interface suggested the formation of artifacts, likely due to heat and recrystallization effects, which obscured the actual ultrastructure.

Table 1 compares the key characteristics of three cutting techniques: Ion Milling, Laser Cutting, and Focused Ion Beam (FIB) milling.

**Table 1** Comparison of key features for Ion Milling, Laser Cutting, and Focused Ion Beam (FIB) techniques for preparing Euplectella aspergillum Spicule cross-section

| Feature | Ion milling | Laser cutting | Focused Ion Beam (FIB) |
|---|---|---|---|
| Precision | Moderate precision, generally less than FIB and laser cutting | High precision (micrometer scale), but less than FIB | Extremely high precision (nanometer scale) |
| Material removal | Sputtering, which can be uneven depending on material properties | Vaporization of material, less controlled | Layer-by-layer, highly controlled |
| Thermal damage | Minimal direct thermal damage, but ion bombardment can induce some heating | Significant thermal damage due to localized heating | Minimal |
| Surface finish | Can be rough, depending on the material and milling duration | Rough, with possible heat-affected zones | Smooth, ideal for high-resolution imaging |
| Speed | Moderate, faster than FIB but slower than laser cutting | Fast, depending on material thickness | Relatively slow, due to the high precision required |
| Cost and complexity | High cost, requires specialized equipment and expertise | Moderate cost, more accessible technology | High cost, requires specialized equipment and expertise |

## Conclusion

This study provided a comprehensive comparison of three advanced techniques—Ion Milling, Laser Cutting, and Focused Ion Beam (FIB) Milling—for the preparation of sponge spicule cross-sections aimed at high-resolution imaging. While ion milling and laser cutting were effective in various material science applications, their use in preparing delicate biological structures like spicules introduced significant challenges. Ion milling caused surface damage and uneven material removal. Laser cutting, on the other hand, introduced thermal damage and micro-cracks. Both of these issues compromised the structural integrity of the spicules and obscured fine details essential for accurate analysis. In contrast, FIB milling emerged as the most effective technique, providing smooth, artifact-free cross-sections with minimal thermal and mechanical stress. The precision and control offered by FIB milling, along with its real-time imaging capabilities, made it the preferred method for preparing fragile biological specimens similar to Euplectella aspergillum spicules. The findings of this study have important implications not only for the sample preparation of marine sponges but also for the broader application of these techniques in biomimetics and materials science, where preserving the integrity of complex structures is essential.

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