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A New and Practical Method for Measuring Sponge Spicules

(Metode Baru dan Praktis untuk Pengukuran Spikula Sponge)

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Abstract

Binocular light microscopy (BLM) is an excellent match for a scanning electron microscope (SEM) and a trinocular light microscope equipped with a micrometer (TLM). The practicality, user-friendliness, and short-time analysis of BLM make this method a good choice for spicule analysis. However, its effectiveness and accuracy are yet to be confirmed. This study aimed to validate the effectiveness of BLM by comparing its usefulness to both TLM and the gold standard methods. BLM was first subjected to measuring megascleres and microscleres of 2 sponges. Then, by using the If function built-in Excell and t-test in SPSS 16.0, the compatibility of BLM was evaluated against SEM by measuring the length of spicules from 4 Sangihe sponges and their counterpart species from different locations. Furthermore, the t-test analysis was used to validate the compatibility and effectiveness of our method to the TLM by measuring the spicules of four sponges. Both the F-function and the t-test analysis proved BLM was compatible with SEM with both measurements showing a perfect match for megascleres typed spicules of 4 compared sponges. This new technique also showed a perfect match with SEM (p = 0.367, t-test) and with TLM (p = 0.963, t-test).

Keywords: Spicules, sponges, SEM, Wallacea, biomaterial, sponge taxonomy

INTRODUCTION

Sponge spicules continue to attract considerable attention because of their biological, environmental, and biomedical potential (Venkatesan et al., 2016). Despite the emerging DNA-barcoding in the identification of sponges (Vargas et al., 2012), metabolomics, and other advanced technologies, a morphological analysis focusing on spicules remains the main method for the sponge taxonomic identification (J. N. A. Hooper & Van Soest, 2002). According to the World Health Organization (WHO), sponge identification plays a key role in ecology, evolutionary, systematic, and biodiversity studies, significantly contributing to the development of conservation and management plans (FAO, 2020). Thus, research in sponge spicule is a cornerstone in sponge taxonomy, which in turn may contribute to marine conservation, protection, and sustainable practices. In addition, the silica structures made by sponges were also reported to have superior optical and interesting mechanical properties (Sundar et al., 2003). For instance, the spicules of the sponge *Euplectella aspergillum* were reported as having fiber-optical characteristics resembling those of commercial telecommunication fibers (Aizenberg et al., 2005). More recently, sponge spicules were also reported as having potential in cosmetics as a skin dermabrasion tool and in biomedical practice as a promising drug delivery system (Tansathien et al., 2019) and as bone substitutes in tissue engineering (Granito et al., 2017). These suggest that the sponge spicules offer

many advantages to modern society, ranging from taxonomy to environmental and marine protection to biomedical and biomaterial potentials.

Despite the promising potentials, however, research on sponges and their spicules remain limited in developing countries, ironically many of which host rich marine biodiversity. One major problem may root in limited access to research instruments such as the scanning microscope electron (SEM) for researchers in developing countries. Over the years, SEM has become the gold standard in the measurement of sponge spicules because this powerful technique provides greater detail of spicules' size and picture and has been proven for sponge taxonomic research (Anđjus et al., 2016). However, there are some limitations to its use including (1) high cost in maintenance and operation, (2) the requirement for highly trained operators, and (3) limited availability especially in many research institutions in developing countries (Acharya & Pathak, 2019). Consequently, sponge taxonomic identification has been scattered and fragmented even in the rich biodiversity regions such as the Wallacea region in Indonesia, leading to the lack of taxonomic knowledge and the exclusion of sponges for decades from coral reefs biodiversity survey and monitoring programs. (Bell, 2008; Bell & Smith, 2004). To overcome these issues, we were interested in developing a new, practical and low-cost technique for measuring sponge spicules that may open up opportunities for researchers particularly from developing countries to research sponges or other important marine invertebrate containing spicules, which may, in turn, contribute to marine biodiversity and environmental protection, biomedical and biomaterial discoveries as well as sustainable marine practices.

In this study, we want to showcase the use of our new, low-cost, and practical technique to identify and measure sponge spicules. First, we developed a formula and a new method for measuring both microscleres and megascleres typed spicules to evaluate the spicules of 2 Sangihe marine sponges. Because the length of spicules is mainly used for classifying either microscleres or microscleres spicule, then we further confirmed the compatibility of our method to SEM by measuring and comparing the mean length of the spicules from 4 Sangihe sponges measured by BLM against the reported data of 4 counterpart species measured by SEM from various locations in the world. However, the fact that the size of spicules may vary greatly due to environmental factors (Cunningham, 1984; Matteuzzo et al., 2015) , minimizing such factors by measuring the spicules of the same sponges will give a more compatible result between BLM and standardized methods. Therefore, we further compared the mean length of spicules from the same 4 Sangihe sponges measured by BLM and the trinocular light microscope (TLM) equipped with a measuring device to validate our method.

METHODS

Samples

In total 8 sponges were collected by SCUBA in 2 and 4 locations in Siau and Sangihe Islands respectively (Figure 1). Of these, we measured the spicules of the 8 specimens by BLM and 4 of them by TLM. Soon after collection, the samples were transported to the laboratory in Politeknik Negeri Nusa Utara (Polnustar) each in a separate plastic bag, and fragments from each sample were taken for spicule preparation and skeletal structure analysis. The data on the length of spicules measured by SEM, however, were obtained from reported values for sponges from various locations around the world including Okinawa Japan(Hoshino, 1985) , the Wallacea region Indonesia (Balansa et al., 2020; Eder et al., 1999; Riyanti et al., 2020; Sapar et al., 2013). New Caledonian (Levi et al., 1998), The Great Barrier Reef and Northern Australia, and the Central West Atlantic Region (Alvarez et al., 1998).

Spicule Preparation

The acid digestion technique was used to dissolve the sponge's tissue as described by (J. N. A. Hooper & Van Soest,

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2002). Briefly, 1 cm sponge fragments were dried in the oven at 105°C for 1-2 h to completely remove water. Then, the fragments were individually placed into a Petri dish containing 5 mL commercial bleach containing 5% sodium hypochlorite. The dried sponge material (0.5 to 1.0 g) was macerated in 5.0 mL Bayclin (15 min. to 2 h), washed four times with distilled water, and rinsed with alcohol (70%) to remove organic materials. Following the maceration, the free spicules of each sponge were mounted on a microscope slide, air-dried for a few minutes, and observed under a light binocular microscope (Olympus, XSZ107BN) with 16x and 40x magnification for the ocular and objective lens respectively. All spicule's pictures were taken by Samsung J4+, whose rear was equipped with an autofocus setup and 13-megapixel camera, through the microscope eyepiece. The length (L) and width (W) of every spicule was determined by dividing its initial length (L1) or width (W1)—measured by the Corel Draw X6 (64-Bit) in cm on the spicule's picture taken previously by the Samsung camera—by the total magnification value of the microscope (640 total magnification) and then multiply the value by 10,000 to get the final value of the measurement in micrometer (µm) with the following detail procedure as follows.

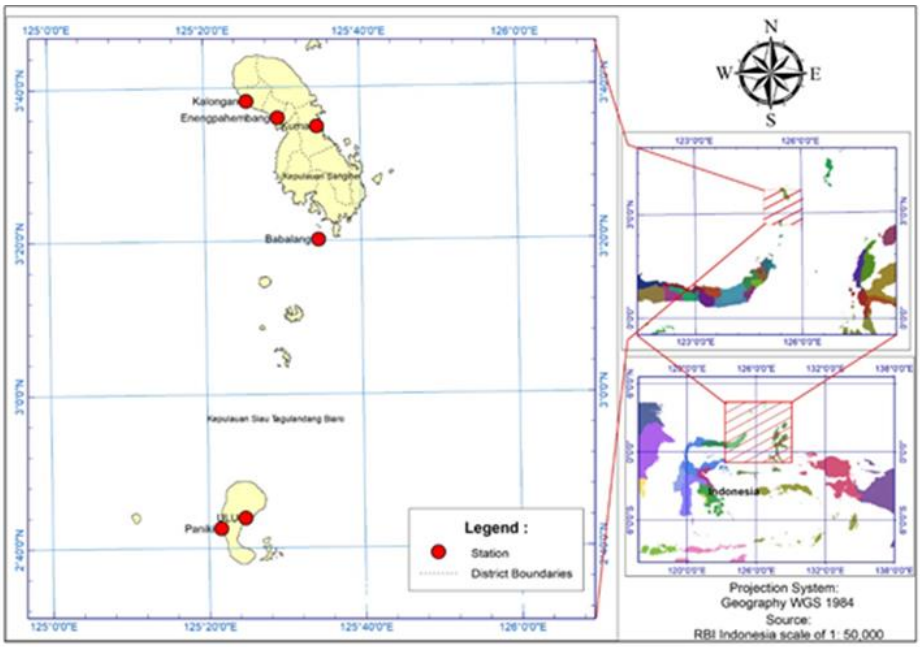

Figure 1. The map for sampling location

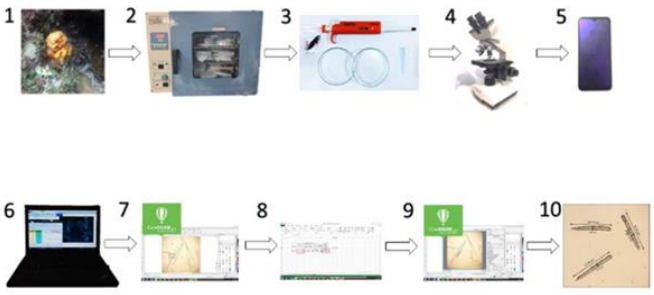

Figure 2. Schematic overview of BLM method for measuring sponge spicules, showing (1). One of the source sponges. (2) Drying process of targeted sponges for 15 m to 1 h in the oven at 150 0C. (3) Maceration of the dried sponges in commercial bleach (Bayclin) for 15 minutes. (4) Observation of spicules under the light microscope. (5) Obtaining images of spicules by a mobile phone. (7) Images transfer and measurement in Corel Draw. (8) Conversion to microscleres in Microsoft excel. (9-10) Final size of the spicule in micrometer.

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t-Test

The parametric statistic, the Independent Sample *t*-test, was used to evaluate the sponge spicules measured by BLM and the standardized methods such as SEM dan TLM. This aims to evaluate the difference or similarity between measurements by BLM and SEM as well as by BLM and TLM. In this analysis, if the t-test showed a *P* value less than 0.05 (p<0.05) or Sig<0.05), this means that there is a significant difference between BLM and SEM as well as between BLM and TLM. On the contrary, if the t-test showed a *P* value larger than 0.05 or (p>0.05) or a significant value larger than 0.05 Sig>0.05), then this means that there is no significant difference between the methods. The *t*-test was performed using SPSS 16.0 with the following mathematical formula.

$$T = \frac{X1-X2}{\sqrt{\frac{(n1-1)S_i2+(n2-1)S_i2}{n1+n2-2}\left(\frac{1}{n1}+\frac{1}{n2}\right)}} \quad \ldots\ldots\ldots 1$$

Where
X1: Mean from the first sample group;
X2: Mean of the second sample group
n_1: n1: The size of the first group
n_2: n2: The size of the second group
S_1: S1: Standard deviation of the first sample
S_2: S2: Standard deviation of the second sample

The IF Function

To make a logical comparison between the size of microscleres and megascleres spicules that are matched or unmatched, we used the following 'IF Function built-in Excell,

=IF(AND(cell for BLM data>=60; cell for SEM data>=60); "Matched"; “Unmatched")

RESULTS AND DISCUSSION

Measuring Megascleres and Microscleres Spicules

Data obtained in previous studies using a scanning electron microscope (SEM) indicated that the morphology and size of spicules from *Agelas nakamurai* as megascleres (>60 mm) acanthostyle, typified by subsequent whirls with a dull and a sharp end at the opposite tips of the spicules (Hoshino, 1985). According to the author, the yellow to red brick in color, tough and resilient in texture, and massive in growth form sponge had the mean length and width of acanthostyle spicules ranging from 185x8 to 226x12 to 267x15 □m, therefore classified as megascleres acanthostyle spicules (see detail assignment in Table 1).

In our study, the size of the spicules of Sangihe *Agelas nakamurai* was first measured in the Corel Draw and later converted into micrometer size (Figure 3A-3C) using the following formula.

$$L = \frac{L1\ x\ CV}{TM} \quad \ldots\ldots\ldots\ldots\ldots 2$$

$$W = \frac{W1\ x\ CV}{TM} \quad \ldots\ldots\ldots\ldots\ldots 3$$

Where
L= Final length in µm
L1= Initial length in cm (measured in Corel Draw)
W = Final width in µm
W1= Initial width in cm
TM= Total Magnification (16 ocular x 40 objective = 640)
CV = Conversion value from cm to µm (10,000)

Figure 3B shows the results obtained using the Corel Draw as 6.7699x0.6297, 6.3091x0.72 and 7.1296x0.6595) cm and width of (0.6297, 0.72 and 0.6596) cm. Plugging these numbers into the above-mentioned formula resulted in the conversion of the length to (105.76, 98,56 and 111.40) □m and width to (9.84, 11,25 and 10.31) □m respectively (Figure 3C and Table 1). Although the values obtained by BLM measurement was smaller than the values reported for SEM, the results obtained by BLM were in good agreement with the reported values for SEM, megascleres (>60 mm) acanthostyle spicules for (Figure 3A-C and Table 1), therefore strongly suggesting the compatibility of our method in measuring megascleres sized spicules. For instance, when measuring the spicules of the Sangihe *Agelas nakamurai* with BLM, we obtained the length of 91.0 □m to 91.9 □m [16-19] providing compelling evidence for megascleres (>60 □m) typed spicules. The results are consistent with the existing data

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for the size of acanthostyle spicules of *A. nakamurai* ranging 5from and 90 □m to 375 □m (Hoshino, 1985).

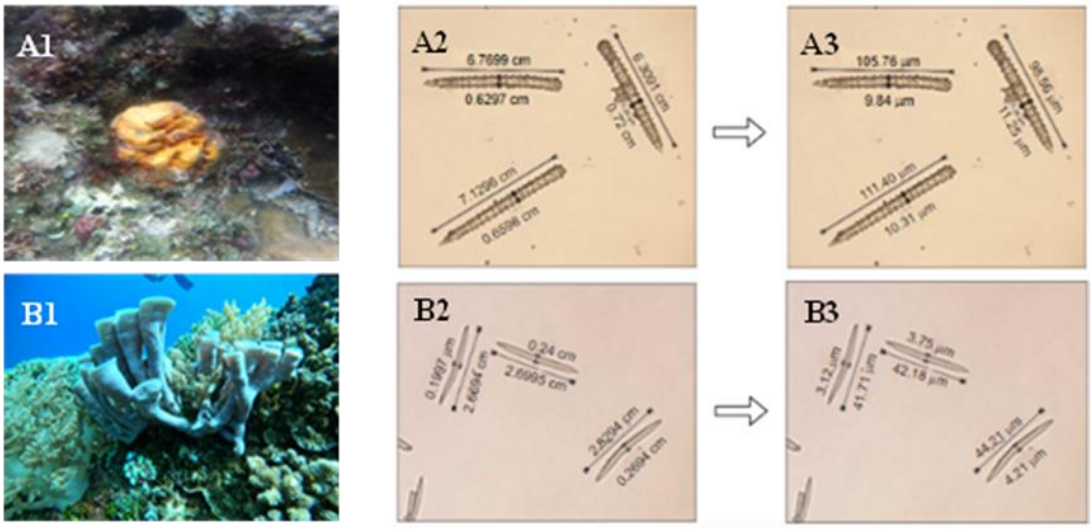

Figure 3. Sponge *Agelas nakamurai* (A1), acanthostyle spicules measured by Corel Draw (A2), and conversion values in micrometer (A3), Sponge *Haliclona fascigera* (B1), microscleres oxea measured by Corel Draw (B2), conversion value to micrometer (B3).

Table 1. Megascleres spicules of *Agelas nakamurai* and microscleres of *Haliclona fascigera*.

Sponge Species	Initial Length and Width Measured by Corel Draw		Magnification		Total Magnification (2x3)	Conversion Value (CV) from cm to µm	Result (µm) (1/4)x5		Category
	1		Objective Lens	Ocular Lens			6		
	L1 (cm)	W1 (cm)	2	3	4	5	L (µm)	W (µm)	
Agelas nakamurai	6.77	0.63	40	16	640	10000	105.78	9.84	Megascleres
	7.13	0.66					111.40	10.31	Megascleres
	6.31	0.72					98.58	11.25	Megascleres
Haliclona fascigera	2.67	0.20					41.71	3.12	Microscleres
	2.83	0.27					44.21	4.21	Microscleres
	2.70	0.24					42.18	3.75	Microscleres

However, the IF function analysis showed a perfect match between spicules measured by BLM and by SEM. We further statistically confirmed the result with the t-test independent sample analysis on the mean length of the spicules of 4 sponges measured by BLM and SEM, resulting in a P-value of 0.327 (P>0.327, t-test) or significant value (sig. >0.327, t-test) (see the detail at Table S3 and Table S4, Supporting Information). The results strongly indicated that, despite the likely effects of environmental factors and geographical locations, there was no difference in spicule measurements by our new method (BLM) and the gold standard method (Table 2), thus proving the compatibility between BLM and SEM.

Nonetheless, the comparison between BLM and SEM was based on the data of spicules isolated from the same species of sponges collected from different

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geographical locations and marine environments (Sangihe sponges vs. sponges from different part of the world). This begged the question of whether or not the absence of environmental and geographical factors would give a better result. Because of the absence of SEM in our region, we decided to compare measurement by BLM and TLM, which is also equipped with a standardized measuring device, in measuring the length of spicules of four Sangihe sponges. Sponges morphological and spicular characteristics are provided in Table S2 (Supporting Information).

Comparing BLM and TLM Methods

Table 3 shows the results gained from measurements by BLM and TLM methods on four sponges. The If function showed 100% matched among all specimens. More importantly, t-test analysis on the independent sample using our method (BLM) and TLM showed a p-value of 0.964 (P>0.963, t-test) or a significant value of 0.964 or (>0.964, t-test) (Table S3, S4, Supporting Information). The result strongly indicated that there was no difference in the measurement results obtained from both BLM and TLM. Also, the result showed that the measurement between BLM and TLM (p = 0.964, t-test) was much more compatible than the measurement between BLM and SEM (*p* = 0.363, t-test). Indeed, statistical data analysis proved the compatibility between BLM and SEM despite the plausible effects of the marine environment, biology, or geography on the size of the spicules of the sponges.

Also, we noticed an improvement in the result between BLM and TLM in comparison to the result between BLM and SEM. As described before in the result and because of various contributing factors that affect the size of spicules, it was crucial to answer the question of whether the absence of environmental, geographical, and biological factors would give better results for two methods being compared to measure the length of spicules from the same sponges, therefore being more compatible.

Table 3. The length of spicules of four sponges collected from Sangihe Islands measured by method BLM and by TLM

Sponge species	Length (in µm)		Type and size of Spicules Microsleres (<60 µm), Megaslceres (>60 µm)		Status
	LM	TLM	BLM	TLM	
X. testudinaria	306,25±23.91[a]	315,26±19.78[a]	Megascleres oxea	Megascleres oxea	Matched
Haliclona tubifera	65,94±5.86[a]	67,90±5.81[a]	Megascleres oxea	Megascleres oxea	Matched
Axinella corugata	487,19±38.60[a]	492,92±64.91[a]	Microscleres oxea	Megascleres oxea	Matched
Neopetrosia sp.	127,50±37.56[a]	136,14±35.46[a]	Megascleres oxea	Megascleres oxea	Matched

Superscript in the Table shows no difference measurement results between the two methods (p>0.05 or sig.>0.05)

Comparing Table 3 and Table 2 shows that both measurements had different p values, being 0.327 (p>0.327, t-test) for BLM and SEM but 0.964 (p>0.964, t-test) for BLM and TLM. This statistically suggests that there is more reasonable compatibility between the results obtained by BLM and TLM in comparison to those attained by BLM and SEM. It is important to note that slightly different results (good results were obtained in the first case and better results in the second case) relate to the use of the same sponges for measurement by BLM and TLM while the same species of sponges from different locations for BLM and SEM. This answers

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our hypothesis that the results from measuring the spicules of the same specimens give more compatible results compared to those of the standardized method. It should be noted that in the second case (the measurement by the BLM and TLM), the use of the same sponges as the source of spicules affected the results, showing a higher *p*-value (p = 0.098, t-test) for BLM vs TLM than BLM vs SEM (p = 0.327, t-test).

Moreover, as described earlier, there seems to be a discrepancy in the type of spicules reported for *Spechiospongia vagabunda* and that of Sangihe *S. vagabunda.* Although this is not the focus of this research, it is worth mentioning that apart from affecting the sizes, the marine environment is also known to affect the type of sponge spicules. Rützler et al., (2014) argued the role of silicon in dictating the change from styloid to tylostyles or subtylostyle typed spicules. Also, while the difference in standard deviation between BLM and SEM was profound (Table 2), it was insignificant between BLM and TLM (Table 3) because the latter method measured the spicules from the same specimens rather than from the same species of sponge but from different environment.

In addition, whereas sponges living in an environment with high silicic acid content could phenotypically express several types of spicules, the same expression was missing from sponges growing in the environment with low silicon concentration.26 This may explain the discrepancy in the typed of spicules in Spechiospongia vagabunda. In addition, the widespread standard deviation of the data was mainly associated with the category of sponge spicules. It is important to reiterate that the size of sponge spicules was divided into two categories, microscleres (<60 □m) and megascleres (>60 □m). Because the size of spicules can vary widely from 60 □m to 3 m (Wang et al., 2020) depending on the species and due to many factors mentioned above, then such a wide range variation in spicule sizes and the standard deviation is expected.

Furthermore, although our hypotheses were supported statistically, several aspects need to be improved in the future. First, the comparison between BLM and SEM was made on the same species of sponges from different geographical locations and marine environments. This affected the measurement results as can be inferred from different *p* values obtained from BLM vs SEM measurements (p =0,327, *t*-test) compared to p = 0.964, *t*-test for BLM and TLM. Hence, it is important to measure the size of spicules from the same specimens by BLM and SEM and compare the results. Also, the number of sponges evaluated in BLM and TLM tests should be increased in future studies. Thus, future work should include more species (at least 10 specimens) designed to evaluate the effect of sample size on the compatibility of BLM and SEM.

Finally, prior work has reported the effectiveness of the gold standard, the scanning electron microscope (SEM). Virtually almost all reported spicular measurements for sponge identification were conducted by SEM because of its detail, high quality, and accuracy, resulting in hundreds of publications such as in the Systema Porifera Book (Hooper & Van Soest, 2002). Unfortunately, the last fifty years has witnessed that almost all sponge identification studies in rich biodiversity countries including Indonesia have come mainly from developed country researchers (Acharya & Pathak, 2019; Calcinai et al., 2017; Voogd & Soest, 2002). In part, this research productivity owed its success to the well-funded research and the availability of research infrastructures in the developed countries (Acharya & Pathak, 2019). In contrast, many researchers in developing countries are still faced with problems such as undersized laboratory infrastructures, inadequate budget as well as limited equipment and reagents (Acharya & Pathak, 2019), unavoidably hampering their research productivity and suggesting the current low contribution of Indonesian researchers in sponge identification efforts. In this research, however, we evaluated the potential of

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simple, low cost and rapid techniques in measuring sponge spicules called BLM. Our method offers several advantages including (a) the practicality of the method as it only requires researcher(s) who can use a light microscope, take the picture through the light microscope eyepiece and use the Corel Draw program, (b) small consumption of reagents and samples (10 mL of 70% alcohol, 2-5 mL Bayclin, 1 cm specimen), (c) short time of analysis (1 hour to run 1 sample), all of which are lacking in the current gold standard method. To run one sample using SEM in Indonesia, it would take 3 days and cost $57,15 per sample. Hence, our new method may open up opportunities for scientists from developing to get involved in research on sponges or other important marine invertebrates containing spicules.

CONCLUSIONS

We showed that our new, practical, user-friendly, low-cost, and rapid technique (BLM method) was very compatible with and as effective as the standardized methods such as SEM and TLM in measuring sponge spicules. Because sponge spicules are known to have important biological, environmental, and biomedical implications, then this new technique has the potential in providing a good ground for further scientific work especially for researchers working in rich marine biodiversity but poorly explored regions to participate in sponge taxonomic identification, bioactive molecules, biomaterial, biomedical discovery as well as in marine conservation and biodiversity protection programs and sustainable marine practices.

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