The use of the SkyScan 1172 high-resolution micro-CT to elucidate if the spicules of the “sea slugs” (Mollusca: Nudibranchia, Opisthobranchia) have a structural or a defensive function

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Aims

The “sea slugs” belong to the Nudibranchia, a group of molluscs (opistobranch gastropods) without shell. Most of them are beautiful tiny colored animals, either to imitate the habitat where they live and/or to warn about their toxicity to their potential predators. Many species have hard, and more or less acicular-shaped, structures (spicules) embedded in the external body wall (including the gills and the chemoreceptive tentacles or rhinophores). The spicules may disappear during the development lacking in adults and being presents only in the larval stages. With respect the origin, Ros (1976) proposed that spicules must correspond to a reminiscence of the typical in origin shell that mollusc species have. However with respect the possible function there is a controversy, existing two main hypothesis considering that the function may be either defensive, complementing the chemical defenses (i.e.: Thompson, 1960; Ros, 1976, 1977; Thompson & Brown, 1976; García et al., 1986; Cattaneo-Vietti et al., 1993; Wägele & Willan, 2000), or structural (Ros, 1976; Vayssière, 1901; Cattaneo-Vietti et al., 1993, 1995). Todd (1981) suggested that the presence of spicules decrease the energetic value of these animals, so they would not be desirable as preys.

Figure 1: In vivo appearance of the “sea slug” species Polycera quadriilineta.
Figure 2: General and detailed lateral view (under the light microscope) from the *in-vivo* appearance of the fore region of the “sea slug” *Polycera quadrilineata*. Spicules are clearly visible by transparence and present along the body, including the rhinophores (marked with red arrows).

To add complexity to the problem, the shape, as well the spicule size varies not only by comparing different species, but also along the life cycle within the same species. Thereafter, it is clear that none of hypotheses are well supported and the problem is still no solved. Thus, to be able to clarify the situation looking for satisfactory evidences, we decided to use the possibilities of the micro-CT techniques to undertake a detailed study of the body structure of the species *Polycera quadrilineata* (Müller, 1776) (Fig. 1). The species has numerous spicules, clearly visible under the light-microscope as quite big, with several axes, and sharpened (Fig. 2).
**Method**

One specimen that had been killed by cooling it (not freezing) in a fridge, and preserved in 70% ethanol to kept it a collection was used for the micro-CT study. Before to carry out the scanning, it was dehydrated by the standard procedure of introducing the specimens for 2 hours in each of a series of ethanol solutions (with increasing percentage of concentrations: 80, 90 and 100%, respectively). After that, it was submerged for 2 hours in Hexamethyldisilazane and overnight air dried.

Animals were scanned with the micro-CT SkyScan 1172 C, by conducting an oversized scan (Number of connected scans=4) with the following parameters: no filter, Image Pixel Size (μm) = 3.08, Source Voltage (kV) = 42, and Source Current (μA)= 47. Images were reconstructed with ©NRecon software, with a Smoothing kernel=2 (Gaussian). Later on, reconstructed images were “cleaned” with ©CT-Analyser by running a Custom Processing Task List (Thresholding, Despeckle, ROI-Shrink-wrap, Reload, Bitwise operations and Save bitmaps), obtaining a new series of reconstructed images. Images of the resulting series were corrected in their position with ©Data viewer, and saved as a definitive series, and finally the volume reconstruction images were obtained with the new volume rendering software ©CTvox. Spicules where pointed out by increasing transparency, depressing the “opacity” curve in ©CTvox; the colours where obtained playing with R,G,B channels, shadows, color material, and light position within the Lighting options.
Figure 3: Different views of the Micro-CT volume rendering reconstruction of the sea slug *Polycera quadrilineata*: right side (a), dorsal (b), ventral (c) and left side (d).
Results
The species *P. quadrilineata* has numerous calcareous spicules -clearly visible under the light-microscope as quite big, with several axes, and sharp-pointed structures- (Fig. 1). With the micro-CT, it could be obtained very clear and neat images, of the structure and spatial distribution (virtually impossible to get with the light microscope). In fact, it was obtained views, not only of the harder parts (spicules, radule, genitalia, etc) but also from the softer body parts. So that it was possible to clearly distinguish the body wall, gills, rhinofores, and organs of the internal anatomy (Figs.: 3-7). So we will continue to work to publish a detailed about it.

With respect to the spicules, they were colored, and clearly pointed out, by playing with the “opacity” and “lighting options” functions of the CT Vox volume rendering software (Figs.: 3-6). Spicules are presents embedded in the body wall (including gills and rhinofores). Their density is higher in the caudal and fore regions. The caudal spicules are longer and more numerous than in other parts. In the middle half of the body the number and size of the spicules decrease. Moreover examining in details the spicules it is clear that the axes have sharpened tips pointing outwards, but not inwards (Fig. 5).

Discussion
Trying to solve the controversy, Penney (2006) conducted experiments and observed that the existence of spicules was not a barrier for crabs, eating without any problem the sea slug species *Cadlina luteomarginata* MacFarland, 1966; and accordingly concluded that the function of the spicules must be structural.

However, if the spicules have a structural function, how to explain the fact that some sea slugs doesn't possess spicules, and some as those of the genus *Dendrodoris* are great size species?. Recently (Sánchez-Tocino & Lucas Cervera, submitted) suggested a double simultaneous structural-defensive function.

The fact that the highest density of spicules is located in the caudal and anterior part of the body (Figs.: 3, 4 & 6), and that they are sharpened and pointing outwards, no inwards (Fig. 5), clearly support the idea of a defensive role, especially considering that spicules are not connected or articulated each other. It that is correct, then why in the middle part of the body (where the genital organes are located, an important part of the body to protect!) the
density of spicules decrease? The answers to this come from the behavior that the species exhibits when it suffer an attack of a predator (most of them fish). As soon as the predator is detected, the

![Image](image_url)

Figure 5: Inner views taken from the caudal part of the body looking towards the head. Camera situated close to the hind tip (a), and at 2/3 from the fore tip (b).

sea slug immediately reacts by contracting the body, forming some sort of sphere. To be able to do this, and considering that they have a lot of spicules in the body, it is necessary to have some free space with a lower density of spicules to permit to contract and condense the body. Clearly, when the animal is contracted offer outside a very compact defensive "coat of mail", offering terrible sharpened defensive weapons pointed outwards. So it is not difficult to imagine the defensive role that they have. In fact predators learn immediately that it is better to avoid those “showy” animals. Another evidence of this defensive role is supported by the fact that the zone with higher density and with bigger spicules is the caudal part. If the function of the spicules were structural, then why the caudal zone with smaller diameter, lesser body mass, is so well protected? It is easy to imagine that the animals could at any time to suffer an unexpected attack of a predator from behind, before they could react to form the defensive sphere-shaped position. In that case the predator will immediately experiments a “stab of pain” giving to the sea slug time enough to adopt the defensive position before to suffer another and definite attack.
Figure 6: Spicules and other dense structures, evidencing the conspicuous spicular "coat of mail". Views from the: right (a), dorsal (b), ventral (c), and left side (d).
Figure 7: Internal anatomy view from the right side (a) and left side (b). (By removing part of the external spicules it is possible to see more clearly the internal organs).

Conclusion
The micro CT technique resulted a valuable tool to point out the structures, including not only of the harder (denser to X-rays) structures (Figs.: 2, 4 & 6), but also the softer organs of the internal anatomy (Figs.: 5 & 7).
Concerning the existing controversy about the function of the spicules of the “sea slugs”, after the micro-CT study of the sea slugs spicules (shape, distribution, and their position with respect the internal organs, as well) there are no doubts about the defensive role that they have.

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