Cytocentrifugation as an additional method to study echinoderm coelomocytes: a comparative approach combining living cells, stained preparations, and energy-dispersive x-ray spectroscopy

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ABSTRACT

Introduction: Echinoderm coelomocytes have traditionally been investigated through a morphological approach using light microscopy, which relies on the idea of constant cell shape as a stable character. However, this can be affected by biotic or abiotic conditions. **Objective**: To analyze if the consistency in cell morphology offered by the cytocentrifugation method, might be used as a convenient tool to study echinoderm coelomocytes. **Methods:** Cells of *Echinaster* (*Othilia*) *brasiliensis* (Asteroidea), *Holothuria* (*Holothuria*) *tubulosa* (Holothuroidea), *Eucidaris tribuloides*, *Arbacia lixula*, *Lytechinus variegatus*, and *Echinometra lucunter* (Echinoidea) were spread on microscope slides by cytocentrifugation, stained, and analyzed through light microscopy. Additionally, fluorescence microscopy, scanning electron microscopy, and energy-dispersive x-ray spectroscopy were applied to cytospin preparations, to complement the analysis of granular and colorless spherulocytes of *Eucidaris tribuloides*. **Results:** Altogether, 11 cell types, including phagocytes, spherulocytes, vibratile cells, and progenitor cells were identified in the samples analyzed. The granular spherulocyte, a newly-described cell type, was observed in all Echinoidea and was very similar to the acidophilic spherulocytes of *Holothuria* (*Holothuria*) *tubulosa*. **Conclusions:** Cytocentrifugation proved to be versatile, either as the main method of investigation in stained preparations, or as a framework on which other procedures may be performed. Its ability to maintain a constant morphology allowed accurate correspondence between live and fixed/stained cells, differentiation among similar spherulocytes as well as comparisons between similar cells of Holothuroidea and Echinoidea.

Key words: comparative cell morphology; echinoderm physiology; energy-dispersive x-ray spectroscopy; invertebrate immunology; spherulocytes; vibratile cells.

Regardless of the scientific scope (e.g. physiology, environmental monitoring), the identification and classification of echinoderm coelomocytes –the circulating cells present in the coelomic fluid– has been traditionally based on morphological data (Matranga et al., 2005; Arizza, Giaramita, Parrinello, Cammarata, & Parrinello, 2007). Morphological characterization of coelomocytes primarily relies on two techniques: transmission electron microscopy (TEM) and/or light microscopy (LM) (Smith, 1981).

TEM analyses have provided fine structural details and information of cytoplasmic components, providing insights on cell physiology and function (Queiroz & Custódio, 2015; Magesky, Oliveira-Ribeiro, Beaulieu, & Pelletier, 2017). However, such analyses require sophisticated infrastructure, and considerable time must be spent in the preparation of samples. By contrast, based on faster and cheaper procedures, LM allows the observation of cell behavior and detection of morphological traits in living cells (Matranga et al., 2005). With this technique, gross morphology and chemical properties of fixed cells can be investigated (Queiroz & Custódio, 2015; Vazzana, Siragusa, Arizza, Buscaino, & Celi, 2015). To perform this procedure, two methods have been employed. The first one consists of fixing cells in suspension just after their collection (Vazzana et al., 2015), while the second one uses the natural ability of living cells to spread and attach themselves to flat surfaces (Branco, Borges, Santos, Junior, & Silva, 2013).

Light microscopy is an easy and quick method, but it has some disadvantages as well. This procedure is based on cell morphology as a stable character, but coelomocyte morphology can be affected by biotic or abiotic conditions. Phagocytes can switch between petaloid and filiform conformations (Edds, 1977, 1993; Canicattì, D'Ancona, & Farina-Lipari, 1989), while spherulocytes may transit between rounder and more elongated shapes (Matranga et al., 2005). Similarly, temperature can influence the spreading ability of phagocytes (Branco et al., 2013). Due to this morphological variability, the accurate identification of different cell types by LM may be compromised. For this reason, a method capable of maintaining a consistent cell morphology would be preferred.

Cytospin may be defined as a centrifugation technique that uses centrifugal force to attach cells directly onto microscope slides (Rathert, Roth, & Soloway, 1993; Gill, 2013). Cytocentrifuges are commonly used in clinical medicine to analyze cells suspended in low-concentrated fluids (e.g. peritoneal and bronchoalveolar; Bibby, 1986; Fleury-Feith, Escudier, Pocholle, Carre, & Bernaudin, 1987). They are used to spread cells in a single layer, thus concentrating them on a small area and preserving morphological details (Qing-fan, 1986). Although cytocentrifugation dates from the mid-1980s and is a well-established method for cell analyses in clinical medicine, its use is not so widespread among invertebrate scholars, and even less so among echinoderm researchers (Raftos, Gross, & Smith, 2004; Majeske, Oleksyk, & Smith, 2013b). To the best of our knowledge, only three (recent) studies have used cytospin preparations to analyze coelomocyte morphology in Echinodermata (Grand, Pratchett, & Rivera-Posada, 2014; Queiroz & Custódio, 2015; Taguchi, Tsutsui, & Nakamura, 2016).

Considering the inherent advantages of cytocentrifugation, as stated by Qing-fan (1986), and the scarcity of studies using this method to analyze coelomocyte morphology, this work aims to address three main questions: 1) Is cytocentrifugation a satisfactory method to investigate coelomocyte morphology in Echinodermata? 2) Could cytospin slides be used in conjunction with other techniques? 3) Would cytocentrifugation be useful in comparing cell morphology of different echinoderm groups? Considering all analyzed groups, i.e. Asteroidea, Holothuroidea, and Echinoidea, we found eleven cell types, which were observed in both live and stained preparations. Cytocentrifugation was used in combination with other methods (e.g. fluorescence microscopy), providing additional data on the spherulocytes of *Eucidaris tribuloides*. Moreover, this method allowed comparisons between similar

coelomocytes belonging to different classes of Echinodermata.

MATERIALS AND METHODS

Specimen maintenance and bleeding procedures: Five specimens of *Echinaster* (*Othilia*) *brasiliensis* (Asteroidea), *Eucidaris tribuloides*, *Lytechinus variegatus*, and *Echinometra lucunter* (Echinoidea) were collected at Praia Grande (23°49'24" S & 45°25'01" W), São Sebastião (SP), SE Brazil, while five individuals of *Holothuria* (*Holothuria*) *tubulosa* (Holothuroidea) and *Arbacia lixula* (Echinoidea) were collected in the Gulf of Palermo (38°06' N & 13°30' E), Sicily, SW Italy. Brazilian echinoderms were acclimated for one week at 23-25 °C in a running seawater aquarium and Italian species were acclimated at 12-15 °C. *Echinaster* (*Othilia*) *brasiliensis* was fed with dead crabs, sea urchins, or small fish collected at the same locality, while sea urchins were fed with frozen algae, as described by Queiroz (2018). Echinoderms from Italy were fed with commercial invertebrate food (Azoo, Taikong Corp., Taiwan).

Coelomocytes were collected following specific procedures according to the species. Cells from *E. brasiliensis* were collected as described by Coteur, DeBecker, Warnau, Jangoux and Dubois (2002), by cutting the tip of one arm and draining 1 mL of coelomic fluid inside an Eppendorf previously filled with 1 mL of anticoagulant solution (20 mM ethylenediamine tetraacetic acid (EDTA), sodium chloride 460 mM, sodium sulfate 7 mM, potassium chloride 10 mM, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mM, pH 8.2 – based on Dunham & Weissman, 1986). This solution was originally developed for marine sponges, but has shown good results also for echinoderms (Queiroz & Custódio, 2015; Queiroz, 2020). Cells from *H. tubulosa* were collected following the protocol of Vazzana et al. (2015). Briefly, a 2 cm-long incision on the anterodorsal side of the specimens was made using a sterile scalpel, then 3 mL of coelomic fluid were drained into 15 mL falcon tubes containing 3 mL of anticoagulant solution. Echinoid coelomocytes were collected according to Queiroz and Custódio (2015), by inserting a syringe needle preloaded with 0.5 mL of isosmotic anticoagulant solution into the peristomial membrane and 0.5 ml of coelomic fluid was withdrawn from each sea urchin.

Cytological preparations: Live cells were observed just after collection by placing drops of coelomic fluid on a microscopic slides, and covered with glass coverslips. For cytological analyses, live cells were deposited on microscope slides using a FANEN 248 simultaneous fluid removal cytocentrifuge. Firstly, the cell density was adjusted to 1×10^6 cells/mL by dilute coelomic fluid using the anticoagulant solution. Subsequently, 60 μL of the sample (6×10^4 cells) were added in each spot and centrifuged by 5 min at 80 x g. Afterwards, slides were fixed for 45 min in formaldehyde sublimate (Custódio, Hajdu, & Muricy, 2004; Queiroz & Custódio, 2015), stained with toluidine blue (TB) or Mallory's trichrome (MT) (Behmer, Tolosa, & Freitas-Neto, 1976) and mounted using Entellan mounting medium (Merck).

Assays with fluorescence microscopy (FM), scanning electron microscopy (SEM), and energy-dispersive x-ray spectroscopy (EDS) were performed to investigate additional application to cytospin preparations. Granular and colorless spherulocytes of *E. tribuloides* were used as a model to analyze other characteristics of echinoderm coelomocytes. Cells stained with MT were used for FM assays, by analyzing the natural fluorescence of acid fuchsin (excitation: 540 nm (green); emission: 630 nm (red); Sabnis, 2010). For SEM and EDS analyses, live cells were deposited on round coverslips and fixed in formaldehyde sublimate as described above. Afterwards, the coverslips were washed once in Milli-Q water for 40 minutes, air-dried, attached to stubs using small pieces of double-sided tape, and stored at room temperature in a closed container with silica gel. Just before SEM analyses, the coverslips were sputter-coated with a 40-60 nm

thick layer of gold and observed in a Sigma VP (Zeiss) scanning electron microscope. For EDS analyses, the coverslips on stubs were analyzed with no further preparations, with the aid of energy-dispersive X-ray spectroscopy coupled to desktop SEM (Phenon world). The results were shown as the mean percentage ± standard deviation (M ± SD) to each element of five cells, and Student's t-tests (α = 0.05) were used to analyze differences between distinct cell compartments inside the same cell (e.g. Nucleus X Cytoplasm) or similar compartments in different cell types. To reduce possible mistakes, common elements from the glass coverslip found in the analyses, such as oxygen, silicon, magnesium, and sodium were removed from the results. Percentages were then recalculated based on the remaining elemental percentages.

Cell identification: Live cells of all species were identified immediately after collection based on morphological features. Afterwards, we looked for cells with the same aspect in fixed preparations (cytospin, MET, and EDS) considering general morphology and/or tinctorial characteristics. Phagocytes were identified by their filopodial or petaloid cytoplasmic expansions, while progenitor cells and vibratile cells displayed a central nucleus surrounded by a thin layer of cytoplasm, and a remarkable flagellum respectively. In Holothuroidea and Echinoidea, more than one type of transparent spherulocyte was found. Thus, based primarily on live preparations, general cell morphology and vacuole (spherule) shape were herein considered key characters to discriminate subpopulations. Lastly, identification was confirmed following specific literature from each class/species: Asteroidea (Kanungo, 1984), Holothuroidea (Vazzana et al., 2015), and Echinoidea (Johnson, 1969; Queiroz & Custódio, 2015).

RESULTS

Cell observations: Analysis performed on live and stained cells identified 11 cell types in the three echinoderm classes, distributed into four broad categories: phagocytes, progenitor cells, vibratile cells, and spherulocytes (Fig. 1, Fig. 2).

Asteroidea: Only phagocytes were observed in live preparations of *E.* (*Othilia*) *brasiliensis*, showing a central nucleus and a largely vacuolated cytoplasm (Fig. 1A). Our analysis of microscope slides revealed that live cells spread immediately after collection, and that live and stained phagocytes display a very similar morphology (Fig. 1D). Progenitor cells and colorless spherulocytes were observed in stained preparations (data not shown), however, they were not found in living preparations.

Holothuroidea: Five cell types were found in *H.* (*Holothuria*) *tubulosa*: phagocytes (filopodial and petaloid morphotypes), progenitor cells, spherulocytes, morula cells, and acidophilic spherulocytes. Live filopodial phagocytes show a central body with several filopodia (Fig. 1B), while petaloid phagocytes display several lamellipodia (Fig. 1C), and the same general patterns were observed in all cytospin preparations (Fig. 1E, 1F). Progenitor cells show a prominent nucleus surrounded by a thin cytoplasmic layer not always visible in live cells (Fig. 1G), which became more evident in TB stained preparations (Fig. 1K). Spherulocytes had a prominent nucleus and round finely granular vacuoles (Fig. 1H) that showed affinity to methyl blue in MT preparations (Fig. 1L), indicating the presence of mucopolysaccharides in the spherules. Also, we verified that the cells usually called "morula" (Vazzana et al., 2015) are actually coelomocytes containing spherules with irregular profile (from round to more elongated). They also stain with methyl blue in MT preparations, evidencing its mucopolysaccharide content (Fig. 1I, Fig. 1M). Acidophilic spherulocytes bear round spherules, with affinity to acid fuchsin in MT preparations, which indicates a protein-moiety (Fig. 1J, Fig. 1N).
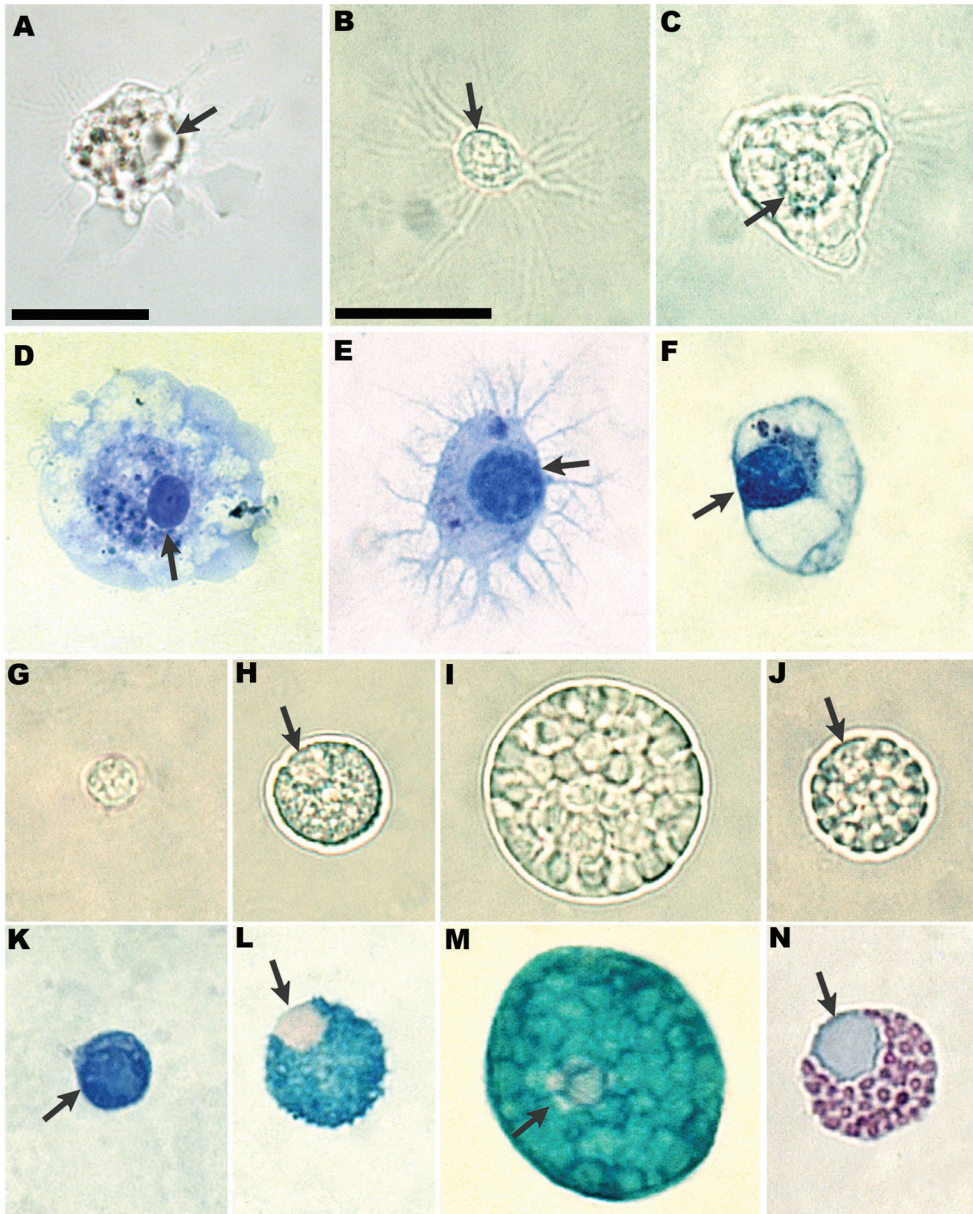
Fig. 1. Live (A-C and G-J) and stained (D-F and K-N) coelomocytes of *Echinaster* (*Othilia*) *brasiliensis* (A and D) and *Holothuria* (*Holothuria*) *tubulosa* (B, C, and E-N). **A-F.** Phagocytes. **G, K.** Progenitor cell. **H, L.** Spherulocytes. **I, M.** Morula cell. **J, N.** Acidophilic spherulocyte. **D-K.** Toluidine Blue. **L-N.** Mallory's trichrome. Arrow = nucleus. Scales: **A.** For cells of *Echinaster* (*Othilia*) *brasiliensis* (10μm), **B.** For cells of *Holothuria* (*Holothuria*) *tubulosa* (10μm).

Echinoidea: Five coelomic subpopulations were observed in all analyzed species: phagocytes, vibratile cells, red and colorless spherulocytes, and one new cell type: the granular spherulocyte. Phagocytes showed a prominent cell body with petal-like cytoplasmic expansions (Fig. 2A'). In cytospin preparations, these cells were irregular in shape, ranging from elongated to roundish, with a subcentral nucleus and a vacuolated cytoplasm

as key features (Fig. 2A, 2F, 2K, 2P). Vibratile cells are round flagellated spherule-filled coelomocytes (Fig. 2B'), which stains light blue in MT (Fig. 2B, 2L, 2Q) or purplish in TB preparations (Fig. 2G), the latter indicating the presence of glycosaminoglycans in the spherules. Live red spherulocytes were readily noticed by the round red spherules present in live cells (Fig. 2C', 2U, 2V, 2W). These cells do not stain in MT slides but present a characteristic brownish color (Fig. 2C, 2H, 2M, 2R), probably due to echinochrome. Even in fixed but not stained preparations, the brownish color is characteristic. Lastly, two types of transparent spherulocytes were identified in Echinoidea, named colorless and granular spherulocytes. Live cells of the first type displayed transparent spherules of variable shape (ranging from roundish to elongated - Fig. 2D'), while live granular spherulocytes showed round uniform spherules (Fig. 2E'). In MT preparations, colorless spherulocytes stained bluish (Fig. 2D, 2I, 2N, 2S), and granular spherulocytes stained pinkish, indicating that their vacuoles are filled with mucopolysaccharides and protein, respectively.

Analysis of granular and colorless spherulocytes of *Eucidaris tribuloides* using FM, SEM, and EDS: Fluorescence analysis of granular and colorless spherulocytes, previously observed in brightfield microscopy (Fig. 3A), showed an intense red fluorescence in the MT preparation due to the affinity of acid fuchsin for granules, which was not observed in the nucleus (Fig. 3B). On the other hand, colorless spherulocytes stained with MT did not show fluorescence (Fig. 3B). SEM analyses allowed a detailed observation of spherulocyte morphology (Fig. 3C, 3D). While granular spherulocytes showed the usual spherical cell shape and regular-sized spherules (Fig. 3C), colorless spherulocytes showed a variable shape, ranging from spherical to more elongated, which was also observed in the spherules (Fig. 3D).

Coelomocyte characterization by SEM was essential to study the elemental composition of the nucleus (Nu) and cytoplasm (Cy) in both cells using EDS (Fig. 3E). For granular spherulocytes, in both nucleus and cytoplasm compartments, the most representative elements were carbon, nitrogen, and sulfur, while phosphorus and calcium were the less common (Table 1). The colorless spherulocyte followed a similar pattern, with some differences. Carbon, nitrogen, and calcium were the most representative elements, and phosphorus and sulfur were the less common (Table 1). Statistical analyses corroborated the differences in elemental composition between distinct compartments in the same cell, or between similar compartments in different cells (Fig. 3E). Thus, the elemental composition of nitrogen, sulfur, and calcium was different between the nucleus and cytoplasm of granular spherulocytes, while sulfur differed in the colorless spherulocyte (Fig. 3E). Comparisons between both spherulocytes showed differences in the composition of nuclear carbon and sulfur, and cytoplasmic carbon, nitrogen, sulfur, and calcium (Fig. 3E).

TABLE 1

Percentage (%) of the main chemical elements in the colorless and granular spherulocytes of *Eucidaris tribuloides*. Values are presented as mean ± standard deviation (M ± SD). Legend: C = Carbon, N = Nitrogen, P = Phosphorus, S = Sulfur, Ca = Calcium

Chemical element	Colorless Spherulocytes		Granular Spherulocytes	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
C	51.90 ± 6.61	57.30 ± 9.18	43.80 ± 3.57	43.70 ± 4.91
N	27.16 ± 5.02	24.80 ± 5.23	27.20 ± 5.97	42.42 ± 5.78
P	3.16 ± 1.66	1.64 ± 1.23	5.76 ± 5.67	0.46 ± 0.30
S	8.12 ± 1.51	3.36 ± 1.56	16.90 ± 4.99	10.34 ± 2.17
Ca	9.66 ± 1.64	12.70 ± 6.88	6.26 ± 2.26	3.08 ± 1.40
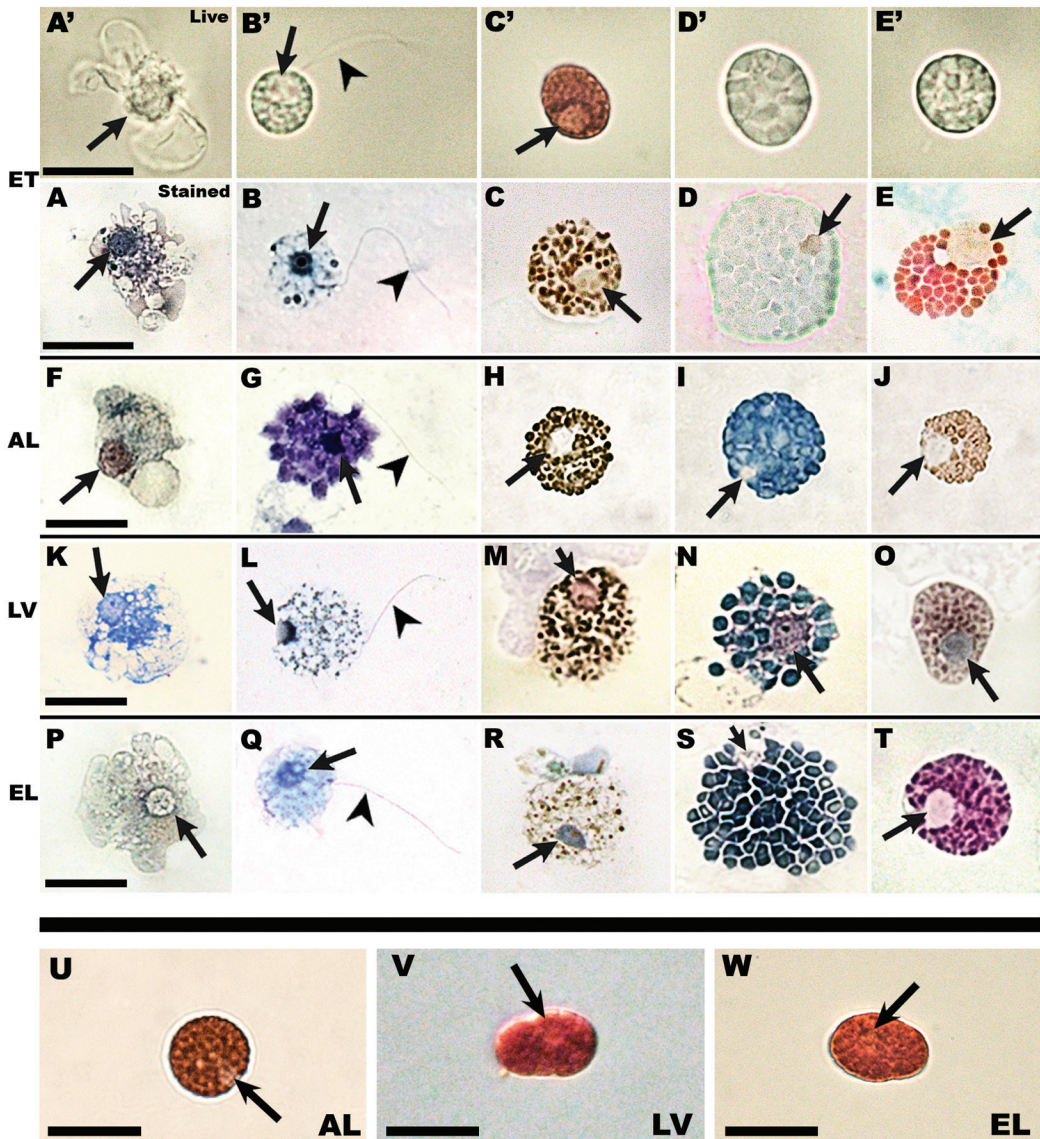
Fig. 2. Live and stained coelomocytes of Echinoidea. **A'-E', A-E.** *Eucidaris tribuloides* (ET). **F-J.** *Arbacia lixula* (AL). **K-O.** *Lytechinus variegatus* (LV). **P-T.** *Echinometra lucunter* (EL). **U-W.** Live red spherulocytes of *Arbacia lixula*, *Lytechinus variegatus*, and *Echinometra lucunter*. **A', A, F, K, P.** Phagocytes. **B', B, G, L, Q.** Vibratile cells. **C', C, H, M, R.** Red spherulocytes. **D', D, I, N, S.** Colorless spherulocytes. **E', E, J, O, T.** Granular spherulocyte. **A-F**, **H-J**, and **L-T**. Mallory's trichrome. **G, K**. Toluidine blue Arrow = nucleus. Arrowhead = flagellum. Scales = 10µm; **A', A.** For live and stained cells respectively of *Eucidaris tribuloides*. **F.** For stained cells of *Arbacia lixula*. **K.** For stained cells of *Lytechinus variegatus*. **P**. For stained cells of *Echinometra lucunter*.
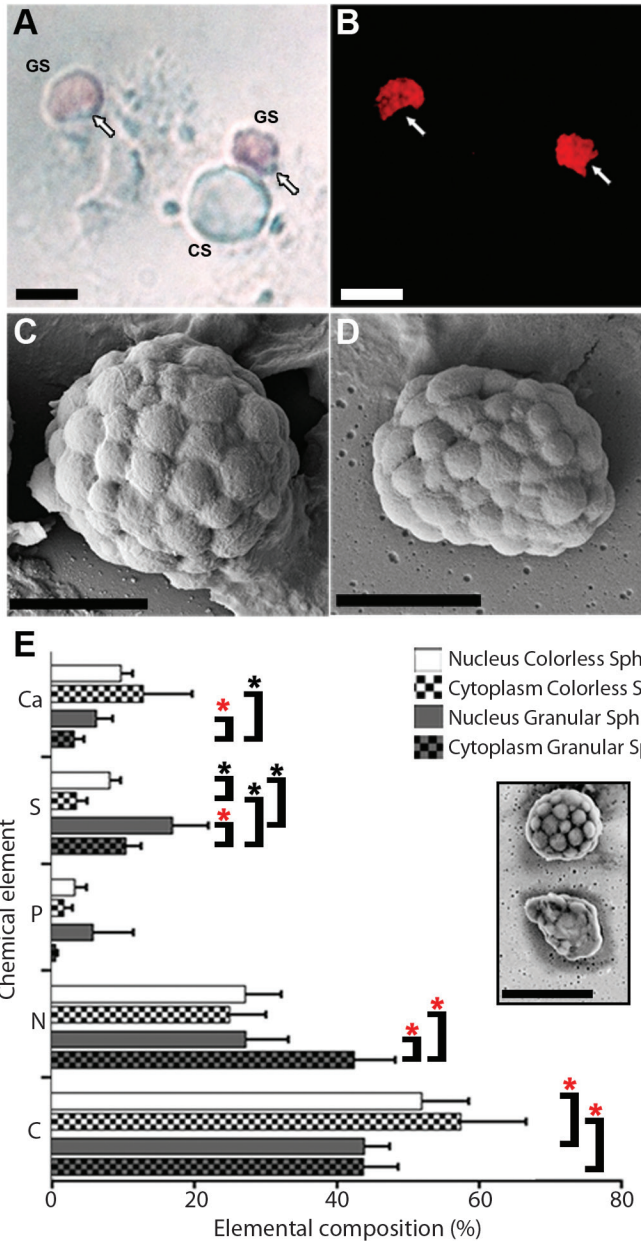
Fig. 3. Granular and colorless spherulocytes of *Eucidaris tribuloides* studied under different methods. **A, B.** Light and fluorescence microscopy of Mallory's trichrome-stained granular and colorless spherulocytes. **C, D.** Detailed morphology of the granular and colorless spherulocytes respectively, under scanning electron microscopy. **E.** Elemental composition of cytoplasm and nucleus of granular and colorless spherulocytes in energy-dispersive x-ray spectroscopy, and morphology of granular and colorless spherulocytes in EDS analysis (*Inset*). C = Carbon. N = Nitrogen. S = Sulfur. P = Phosphorous. Ca = Calcium. CS = Colorless spherulocyte. GS = Granular spherulocyte. Red asterisk = P < 0.05. Black asterisk = P < 0.01. White arrow = Granular spherulocyte nucleus. Scale: A, B, E = 10μm; C, D = 5 μm.

DISCUSSION

In the present study, we confirmed that: 1) cytocentrifugation was satisfactory to analyze coelomocyte morphology in Echinodermata, revealing 11 cell types in the six species analyzed; 2) cytospin slides can be used along with other techniques, providing additional relevant data, and 3) cytocentrifugation allowed us to compare coelomocytes of different echinoderms classes. Considering these results, it can be suggested that cytocentrifugation is an improved method for additional analysis in coelomocyte studies in Echinodermata.

Through cytocentrifugation, coelomocytes are forced to spread onto slides in short runs, binding more efficiently and faster than when allowed to adhere under passive conditions. Incubating cells from suspensions and waiting for them to settle, attach, and spread, has been the most usual procedure to attach coelomocytes on flat surfaces (Bertheussen & Seljelid, 1978; Romero, Novoa, & Figueras, 2016). This poses three main problems: 1) The ability to attach and spread on surfaces is not observed in some coelomic populations (e.g., vibratile cells), which are usually lost during slide processing (Romero et al., 2016); 2) The timespan to achieve cell adhesion may be very long (Romero et al., 2016), which can lead to the degradation and loss of cell characters; 3) The ability to attach and spread on the glass surface, and consequently the cell morphology, can be affected by environmental conditions, such as temperature (Branco et al., 2013). We understand that in some specific situations where the preservation of the natural shape of coelomocytes is necessary (e.g. Edds, 1993; Majeske, Bayne, & Smith, 2013a), cytocentrifugation may not be the best protocol to access cell morphology. However, cytospin preparations are more efficient compared to the usual method, since all cell types can be spread onto common microscope slides without further preparation (e.g. Poly-L lysine; Majeske et al., 2013b), within a short period, and their morphology did not vary considerably.

To the best of our knowledge, only three studies have used cytocentrifugation to analyze echinoderm coelomocyte morphology (Grand et al., 2014; Queiroz & Custódio, 2015; Taguchi et al., 2016), and all of them showed a high potential to improve cell analyses in Echinodermata. Only phagocytes and small spherical cells were previously known to the sea star *Acanthaster planci* (Boolotian, 1962). However, in a study using cytocentrifugation, Grand et al. (2014) described four distinct coelomocytes to this species, showing that its coelomocyte diversity may have been underestimated. Similarly, a new spherulocyte was also detected in *E. tribuloides* (Queiroz & Custodio, 2015), and for the first time, spherulocytes were reported to be able to phagocyte foreign particles (Tagushi et al., 2016). In the same way, we also obtained an unequivocal correspondence between living and fixed cells of all analyzed species, and for the first time, we report a fifth cell type –the granular spherulocyte– in the well-studied echinoids *A. lixula*, *L. variegatus*, and *E. lucunter* (Branco et al., 2013). Thus, our data show that this method can improve morphological descriptions by relating living and fixed cells. It was also able to demonstrate that the newly-described granular spherulocyte (Queiroz & Custodio, 2015) is not restricted to *E. tribuloides*, and may be widespread among regular echinoids.

Another important result obtained herein was the possibility to differentiate spherulocyte subpopulations in fixed preparations. These subpopulations are identified due to their spherule-filled cytoplasm and named after the characteristics of living cells, but are yet a poorly understood cell type in Echinodermata (Ramírez-Gómez & García-Arrarás, 2010). Still, even when alive, spherulocyte identification may be imprecise because more than one type of "transparent" spherulocyte may be present, as we report herein. Consequently, the correct identification of live or fixed cells is still difficult. However, we demonstrate herein that cytocentrifugation is a useful alternative method to discriminate spherulocytes since it allows a good correlation between live and

fixed/stained cells. For example, three different subpopulations were unequivocally identified in live and fixed preparation of *H. (Holothuria) tubulosa*, *E. tribuloides*, *A. lixula*, *L. variegatus,* and *E. lucunter*, and differentiated by cell profile and vacuoles shape (when alive), and its stain affinity (in fixed preparations). By contrast, the correspondence between live and fixed spherulocytes of *Holothuria (Selenkothuria) glaberrima* (see Fig. 2A-C in Ramírez-Gómez, Aponte-Rivera, Méndez-Castaner, & García-Arrarás, 2010) and *Echinometra mathaei* (see Fig. 5 in Piryaei, Ghavam-Mostafavi, Ghavam-Mostafavi, & Pooshang-Bagheri, 2018) leaves some doubts. We strongly believe that correspondence between live and fixed preparations could be increased if cytocentrifugation had been used to prepare the samples in these studies.

Although cellular characteristics obtained by light and even electron microscopy may fail to identify homologous cell types between distinct phyla, these methods might be a useful tool between closer species (Arendt, 2008). Thus, the morphological approach presented here can be used to compare cells from different echinoderm classes, and the acidophilic and granular spherulocytes provide one interesting example. These two coelomocytes are identified by their transparent color and spherule shapes (Vazzana et al., 2015; Vazzana et al.,2018; Queiroz & Custodio, 2015). Both spherulocytes showed uniform spherules filled with a peptidic moiety, as showed by the pinkish color produced by the acid fuchsine present in MT solution. We recognize that even though morphology and chemical contents are similar, further studies are still necessary. Only the discovery of specific cell markers - such as those expressed in helper and cytotoxic lymphocytes (Taniuchi, 2018) - can elucidate this issue. However, the approach used herein can improve the understanding of spherulocyte diversity and can certainly be employed as an additional strategy to understand the real nature of vibratile cells in Echinodermata (Hetzel, 1963; Xing, Yang, & Chen, 2008; Smith et al., 2018).

Cytocentrifugation is also quite useful as a preparation method, alongside which other procedures may be performed, mainly when the aim is to analyze coelomocyte diversity. Some studies used cytospin preparation as a framework for FM (Clow, Raftos, Gross, & Smith, 2004; Majeske et al., 2013b; Golconda, Buckley, Reynolds, Romanello, & Smith, 2019) or SEM (Grand et al., 2014). However, only two of these works (Clow, Raftos, Gross, & Smith, 2004; Grand et al., 2014) analyzed cell morphology using cytocentrifugation. Using cytospins, further aspects of the granular spherulocyte were analyzed herein by different techniques. FM revealed that, in addition to the morphological characteristics, the red fluorescence of the protein-filled spherules may be an identification marker, while SEM corroborated the differences in the shape pattern of cytoplasmic spherules observed in light and fluorescence microscopy.

EDS has been commonly used to analyze the presence of uncommon elements (e.g. heavy metals) in invertebrate coelomocytes/ hemocytes (e.g. Tullius, Gillum, Carlson, & Hodgson, 1980), but few studies have focused on conventional cell elements (Scippa, De Vincentiis, & Zierold, 1990). This method revealed specific differences in the elemental composition in different compartments of the same cell as well as between similar compartments of different cells, which could reflect organelle functioning. For example, sulfur was higher in the nucleus if compared to the cytoplasm of the granular and colorless spherulocytes of *E. tribuloides,* a pattern similar to that observed in the morula cell of the urochordates *Halocynthia papillosa* and *Phallusia mammillata* (Scippa, Botte, Zierold, & De Vincentiis, 1985; Scippa, De Vincentiis, & Zierold, 1993). By contrast, although nitrogen has not been mentioned to other invertebrates (e.g. Scippa et al., 1990; Scippa et al., 1993; Giamberini, Auffret, & Pihan, 1996), this element was higher in granular but not in colorless spherulocytes.

Higher nuclear sulfur can indicate higher levels of Methionine, an amino acid universally associated with the initiation of protein

synthesis (Kozak, 1983). Such an explanation fits properly with the general function of the morula cell of *H. papillosa* and spherulocytes of *E. tribuloides*, which are cells adapted to produce and store chemical compounds. In contrast, higher cytoplasmic nitrogen corroborates the assumptions of a rich-protein moiety observed through acid fuchsin affinity. Lastly, if considered in conjunction, the two-fold levels of nuclear sulfur and cytoplasmic nitrogen observed in granular spherulocytes confirm the engagement of this cell in protein production. Therefore, our results suggest that sulfur and nitrogen concentrations are different in colorless and granular spherulocytes of *E. tribuloides,* and could be used as a specific chemical marker to differentiate these cells in other echinoids. This approach could provide important results in the cells of other invertebrates.

Most studies addressing echinoderm coelomocytes by FM, either using cytospin preparations (Clow, Raftos, Gross, & Smith, 2004; Golconda et al., 2019) or other methods (Brockton et al., 2008; Majeske et al., 2013a), have focused on sea urchin phagocytes, while spherulocytes, in general, have been poorly studied (García-Arrarás et al., 2006; Falugi et al., 2012). That may be due to the ease in identifying phagocyte subpopulations. Even with the use of specific markers to discriminate phagocyte subtypes, the identification is still mostly based on morphology (Golconda et al., 2019). To SEM, although Grand et al. (2014) have used cytocentrifugation to analyze *A. planci* coelomocytes, the authors did not provide a more detailed correspondence among the living, stained, and SEM preparations, which impairs further comparisons. Regarding EDS, as far as we know, there is no work using cytocentrifugation to perform this technique. However, Falugi et al. (2012) used an Environmental Scanning Electron Microscope coupled with EDS to analyze the presence of nanoparticles in smeared cells. In this latter study, which certainly provided important data on the effect of nanoparticles on echinoderm coelomocytes, some additional aspects could have been observed if cytocentrifugation was used. For example, in addition to confirming the presence of Sn, Ce, and Fe inside the coelomocytes, and observing impacts on subcellular compartments, Falugi et al. (2012) could have answered other relevant questions, such as: 1) Do different cell types accumulate nanoparticles equally? 2) Can nanoparticles be found in the nucleus? 3) Which cell type was most impacted at the subcellular level? Consequently, our results show that cytocentrifugation could be a useful tool to improve studies on coelomocyte in Echinodermata and other invertebrates as well.

Cytocentrifugation proved to be versatile, being useful as the main method to investigate echinoderm cell morphology in stained preparations or as a framework for other procedures. Although stained phagocytes displayed little variations if compared to live cells, cell identification was not impaired. Therefore, cytocentrifugation maintains constant cell morphology, which allowed accurate correspondence between live and fixed/stained cells, correct identification of apparently similar types of spherulocytes, as well as comparisons between related species. Still, it is a fast, practical, and inexpensive method, useful to different samples. This includes low concentrated cell suspensions, which would allow the study of species with little body fluid volumes such as Crinoidea and Ophiuroidea. Finally, this method may be used either as a convenient strategy able to improve cell morphology or coelomocyte physiology studies. Thus, the present work provides strong evidence that cytospin preparations have a high potential to improve coelomocyte analyses in Echinodermata and other invertebrates.

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RESUMEN

Citocentrifugación como un método adicional para estudiar celomitos de equinodermos: un enfoque comparativo que combina células vivas, preparaciones teñidas y espectroscopía de rayos-x de dispersión de energía

Introducción: Los celomocitos de equinodermos se han investigado tradicionalmente a través de un enfoque morfológico utilizando microscopía óptica, que se basa en la idea de la forma celular constante como un carácter estable. Sin embargo, esto puede verse afectado por condiciones bióticas o abióticas. **Objetivo:** Analizar si la consistencia en la morfología celular que ofrece el método de citocentrifugación podría utilizarse como una herramienta conveniente para estudiar los celomocitos de equinodermos. **Métodos:** Células de *Echinaster* (*Othilia*) *brasiliensis* (Asteroidea), *Holothuria* (*Holothuria*) *tubulosa* (Holothuroidea), *Eucidaris tribuloides*, *Arbacia lixula*, *Lytechinus variegatus* y *Echinometra lucunter* (Echinoidea) se esparcieron en portaobjetos de microscopio por citocentrifugación, se tiñeron y analizaron mediante microscopía óptica. Adicionalmente, se aplicó microscopía de fluorescencia, microscopía electrónica de barrido y espectroscopía de rayos X con dispersión de energía a las preparaciones de citoespina, para complementar el análisis de los esferulocitos granulares e incoloros de *Eucidaris tribuloides*. **Resultados:** En total, se identificaron en las muestras analizadas 11 tipos de células, incluidos fagocitos, esferulocitos, células vibrátiles y células progenitoras. El esferulocito granular, un tipo de célula recién descrito, se observó en todos los Echinoidea y fue muy similar a los esferulocitos acidófilos de *Holothuria* (*Holothuria*) *tubulosa*. **Conclusiones:** La citocentrifugación demostró ser un método bastante versátil, ya sea como el método principal de investigación en preparaciones teñidas o como un marco en el que se pueden realizar otros procedimientos. Su capacidad para mantener una morfología constante permitió una correspondencia precisa entre las células vivas y las células fijas/teñidas, la diferenciación entre esferulocitos similares, así como comparaciones entre células similares de Holothuroidea y Echinoidea.

Palabras clave: morfología celular comparativa; fisiología de equinodermos; espectroscopía de rayos-x de energía dispersiva; inmunología de invertebrados; esferulocitos; células vibrátiles.

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