# Sea urchin coelomocytes cultured on nanoporous aluminium oxide as a potential tool for marine environmental monitoring

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**Abstract:** Oceans and seas are of paramount importance to the health of planet Earth and humankind. Marine ecosystems should be defended against the attack of polluting agents by continuous monitoring; the means of such monitoring should be as green as possible, i.e. based on sensors manufactured by bio-compatible and easily disposable raw materials. In this study, we lay the basis for the future development of biosensors of the marine environment based on sea urchin cells cultured on nanoporous aluminium oxide. These cells are promising as in previous works they demonstrated high response to stressors, and the proposed substrates have low costs since fabricated by inexpensive anodization process from consumer quality aluminium foils. Coelomocytes of the Mediterranean sea urchin *Paracentrotus lividus* were cultured on the nanoporous alumina for up to 5 days *in vitro*. Then, a biochemical characterization was carried out, checking the cholinergic system pathway by means of serotonin-autofluorescence induced by aldehyde exposure and by expression and functionality of neuroactive molecules, such as acetylcholinesterase and muscarinic acetylcholine receptors. Both living cell quality and system biochemistry were not affected after the culture was carried out, and both electrical modulation and non self-reactivity were maintained. These findings suggest the possibility of using the sea urchin immune cells cultured on nanoporous alumina as tools for monitoring the marine water quality, based on their electrical response.

**Keywords:** nanoporous alumina; anodization; living cell culture; Paracentrotus lividus; cholinesterase; biosensor

## 1. Introduction

Presently, a large effort is being made worldwide to increase the sustainability of human activities, including industrial and agricultural development, while preserving the quality of the environment and of animal and human life (Giovannoni and Fabietti, 2013). One approach is to develop *in vivo* toxicology testing in fields such as environment and food contamination. Generally, vertebrate models are used, but over the past few decades the researchers have been seeking alternatives, in the light of the three Rs principle: replacement, reduction and refinement (Russell and Burch, 1959). One possible choice is using marine invertebrates, at both tissue and cell culture levels, e.g. sea urchin coelomocytes and bivalve haemocytes (Canesi *et al.*, 2005; Falugi *et al.*, 2012). Although these can differ considerably from vertebrates, they appear to be promising sentinels of environmental water quality (Pinsino and Matranga, 2015).

In particular, sea urchin coelomocytes (SUCs) are progenitors of immune cells in vertebrate systems: their life-cycle takes place in the coelomic liquid suspension, same as vertebrate blood cells do in plasma, and they carry out similar functions, such as clot formation, phagocytosis, encapsulation, clearance of bacteria or other foreign materials, and oxygen transport (Matranga *et al.*, 2005). Conversely to culture cells from other marine organisms, SUCs offer the advantages to be directly exposed to seawater without suffering osmotic shock, since they are naturally free-wandering cells dispersed in the coelomic fluid (Glinski and Jarosz, 2000). SUCs respond to the requirements of the three Rs principle, for ease of sampling and scarcely invasiveness of the used specimens, and they have been proposed as good models to assess the seawater quality, thanks to their sensitivity to stress conditions both in laboratory and field studies (Matranga *et al.*, 2006; Pinsino, Torre and Sammarini, 2008). This ability of SUCs has been demonstrated by analyzing their biochemical behavior and environmental-related changes. These cells respond to physical or chemical stress by increasing certain subpopulations (i.e. red amoebocytes (Smith *et al.*, 2010)), and by expressing heat shock proteins (HSP70 (Matranga *et al.*, 2000, 2005; Matranga, Bonaventura and Di Bella, 2002; Falugi *et al.*, 2012)), which are the signals for innate immunity (Tsan and Gao, 2009). Additionally, the presence and function of a complete set of molecules belonging to the cholinergic system (i.e choline acetyltransferase, ChAT; acetylcholine receptors; acetylcholinesterase, AChE) and/or immunologically related to it (i.e. CD41, CDF/LIF molecules) were shown in SUCs of the Mediterranean sea urchin *Paracentrotus lividus*, and were compared to those present in the human blood cells (Gambardella *et al.*, 2015). In human cells, the enzyme activity of AChE is associated to red blood cell membranes, as well as to the perinuclear region of leucocytes, suggesting a role in the function of the latter (Leal *et al.*, 2017). The presence of AChE activity in *P. lividus* SUCs was characterized by specific inhibitors, in relation to cold stress induced in the laboratory (Angelini *et al.*, 2003). The impairment of molecules involved and/or immunologically related to the cholinergic system of SUCs (i.e. AChE, ChAT, CD41/LIF) seems to be related to environmental stress, according to findings on vertebrates (Gambardella *et al.*, 2015). All these features make the sea urchin a good model for transferability to environmental and human health, being a reliable, cost-effective and easy tool for different purposes.

Few attempts have been done so far to propose working procedure able to assess long-term SUC viability and survival over long periods. To our knowledge, the only available data on SUC cultures refer to short-term cultures on traditional substrates (Dan-Sohkawa et al., 1993; Matranga et al. 2002, 2006; Majeske et al. 2013), where simple and complex media) have been used. The only long-term SUC study has been recently published (Pinsino and Alijagic, 2019) and it used an innovative physiological-like media on traditional substrate. To date, no research is available on SUC on engineering substrates, aimed at achieving a portable biosensor to be used in the environment.

In order to implement a biosensor based on SUCs, a controlled, reliable substrate for living cells adhesion is necessary. On the one hand, this substrate should be fully biocompatible to preserve the cell functionality. Also, the substrate should be prone to integration in a biosensor device, based on either optical or electrical readout. We have identified the anodic porous alumina (APA) to be the appropriate substrate platform for development of such a SUCs-based biosensor. APA is a nanostructured material surface consisting of a pattern of columnar nanopores, which should be biocompatible (Ingham, ter Maat and de Vos, 2012; Brüggemann, 2013; Toccafondi *et al.*, 2015). Its safe interaction with a number of different types of living cells has been demonstrated, even with sensitive neurons (Ingham, ter Maat and de Vos, 2012; El Merhie *et al.*, 2019). APA has also already been the object of a number of exploratory studies for chemical sensors, mainly based on either surface-enhanced Raman spectroscopy (Chiara Toccafondi *et al.*, 2016) and interference spectroscopy (Kumeria and Losic, 2011). Here, we investigate for the first time the effect of using APA as the substrate for cultures of SUCs, for up to 5 days *in vitro* (DIV). The goal is to assess the biocompatibility of APA vs this type of living cells, in view of the development of the mentioned green marine biosensor.

This research activity started in September 2017 and ended in July 2019. Sea urchins were collected in the Ligurian Gulf, Mediterranean Sea, Italy. APA substrates and culture tanks were constructed at DICCA, University of Genoa (ECPLab at Savona-Campus), Italy, where also cell cultures were maintained; SEM imaging was carried out at Italian Institute of Technology IIT (MCF), Genoa, Italy; and sea urchin manipulation and cell visualization were performed at CNR-IAS, Genoa, Italy.

## 2. Materials and Methods

All the reagents were obtained from Sigma IT, or otherwise specified. All the solid surfaces, including APA disks and culture tanks described in the next paragraphs, forceps and pipettes were sterilized in UV chamber (GERMIX XDQ-504) for at least 24 h.

### *2.1. APA substrate*

We used consumer aluminium foil for food (Cuki plates), ~0.2 mm thickness. The foil was cropped to circles of ~3 cm diameter, and first degreased with acetone-wet cotton tissue. Afterwards, deoxidation and desmutting were carried out according to industrial protocols (Runge, 2018), by dipping the discs in 4 M aqueous NaOH for 1 min and in 30% aqueous $HNO_3$ for 30 s, respectively, followed by rinsing with deionized (DI) water at each step. The discs were loaded in a Teflon beaker with a stainless steel plate on the bottom side, providing electrical contact for anodic polarization. The beaker

was filled with 0.3 M aqueous $H_3PO_4$. An external high-voltage DC power source N5751A was used (Agilent, USA). The cathode was a platinum basket, dipped in the beaker from the top and kept at ~2 cm distance from the aluminium. Anodization was carried out without temperature control, under mild magnetic stirring. The power supply was set to both limiting voltage (110 V) and current (1 A). When the circuit was first closed, a sudden current surge occurred, which was cut by the respective limit. In few seconds, the current decreased, and the process turned to potentiostatic mode, according to the limiting voltage level set. The duration was usually 30 min. After anodization, the beaker was emptied and filled with DI water to rinse and cool. The anodized disc was removed and thoroughly rinsed with DI water, and dried with a flow of warm air. The APA surface was cut by means of a punching machine, to form smaller discs of approximately 5 mm diameter.2.2. SEM imaging

From all the fabricated APA substrates, a small portion was cut and inspected under a scanning electron microscope (SEM) to assess the presence of the nanopores for living cell adhesion and check their typical size and spacing. To this goal, we first overcoated the sample with ~15 nm thick gold by thermal evaporations, and then used a SEM model JSM 649OLA (JEOL, Tokyo, Japan), typically at 30 kV acceleration voltage and magnification of 10X, collecting the standard morphological signal of secondary emitted electrons.

*2.3. Culture tanks*

Special culture tanks in polylactic acid (PLA) were designed and constructed by the use of a tridimensional printer. Culture tanks (see Fig.1) are constituted by two concentric compartments both filled with ultra-filtered and pasteurized seawater (UFSW). The APA substrate to be used for the SUCs culture was attached on the bottom of the internal compartment. The external compartment can be exposed to contaminants that move to the culture posed in the internal one by diffusion In order to control the mechanism of diffusion of the culture at different exposure times, a washer made of sterile filter paper is placed on the top of the internal compartment and fixed in its position by the tank cap. This annular profiled porous material allows the pollutant to slowly enter into the internal compartment with null velocity, so as not to risk disconnecting the cells from the porous surface and reaching the culture in diluted concentration. A particular texture was chosen in the fabrication of the culture tanks, in order to produce a semi-permeable material that allows oxygen diffusion from the external air to the compartments, despite being completely impermeable to seawater; oxygen supply has proved to be of fundamental importance for the long-term culture. Another important issue that could assume a significant importance for long-term cultures is the possibility of nutrient supply to the cells. The tank cap is provided by a disposal that can contain glucose in solid phase being slowly released (Fig.1a). The structure of the culture tank is shown in Fig.1b.

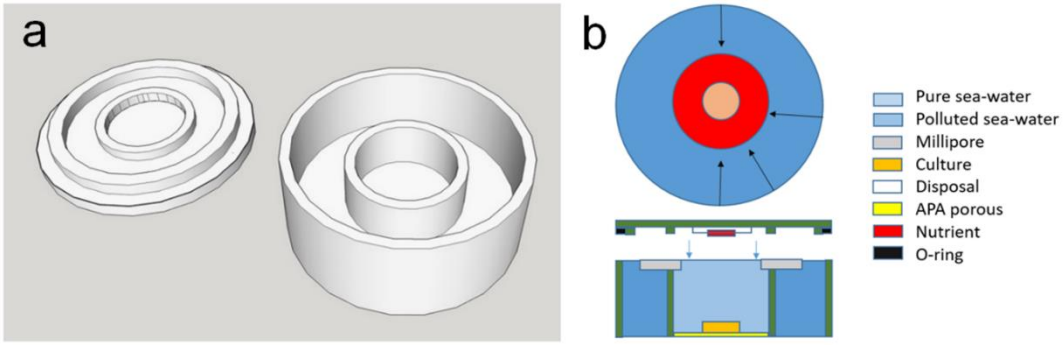


**Fig. 1** Sketch of the tanks used for cell incubation to optimize diffusion. a) 3D tank overview; b) section of the tank with diffusion path (arrows) and description of loading media and accessory components

*2.4. Sea urchin celomocytes (SUCs) preparation*

Adult specimens of the sea urchin *P. lividus* were sampled from July to September from uncontaminated regions of the Ligurian Gulf (Mediterranean Sea, Italy). The adults were carried to the laboratory enveloped in a seawater wet tissue inside a refrigerated bag, according to published protocol (Amemiya, 2001). In laboratory, the samples were re-hydrated, by immersion in UFSW, in order to increase the volume of the coelomic fluid. The UFSW was performed through a 0.22 µm filter (Whatman), by a vacuum pump and it was previously supplied from the Aquarium of Genoa (Italy) at a salinity 37‰. SUCs were sampled by using a sterile syringe (Insumed, ultrafin 29G), immersed through the perioral membrane to reach the coelomic cavity. The coelomic fluid containing the cells of the single specimens was collected in sterile conditions in different Eppendorf vials and immediately used for the cultures. After this procedure, the urchins were still alive and were reintroduced to their natural environment A part of the coelomic fluid was used to count the cell/volume ratio. 15 µL cell-free coelomic fluid, containing about 3500-4000 cells, were deposed on a control disk (aluminium) and on APA disk (5 mm diameter), placed at the bottom of the inner chamber of PLA tanks. After 10 min, when the cells had landed to the surface of the APA disk, 0.5 mL of UFSW-diluted ultra-filtered (Whatman) coelomic fluid were added in the internal chamber of PLA tanks, containing 1:5 and 1:1000 sucrose. A portion of coelomic fluid for each sample was maintained at -20°C for further quantitative biochemical analysis of enzyme activity. The outer PLA chamber was filled with 1.5 mL of UFSW, in order to prevent dehydration and consequent salts concentration. A plug was put on the tank for the whole test to preserve the culture by external contamination. The samples were collected after 2, 3 and 5 DIV, and stained with vital stains in order to check the survival percentage. No solid nutrient was inserted in the cap disposal due to the chosen tests duration.

*2.5. Staining*

2.5.1. Vital stains

To verify cell quality and vitality, SUCs cultured on APA substrate for 5 DIV were stained with 4',6-diamidino-2-phenylindole (DAPI), a dye that selectively stains DNA, by binding to the A-T –groups in both viable and fixed cells (Kapuscinski, 1995). DAPI was dissolved 1:100 in acetate buffer, pH 7. The mother solution was diluted 1:100 in SW and added to the cells, for 30 min in the dark. The coverslips

were rinsed with SW and mounted with glycerol-gel, and coelomocytes were observed under epi-fluorescent microscope (Olympus, Japan).

2.5.2. AChE activity cytochemical localization

To localize AChE after 5 DIV culture, the SUCs on the APA disks were drawn by use of a glass pipette, posed on a glass slide coated with glycerinated albumin, immersed in 0.2% glutaraldehyde in UFSW, rinsed, and stained according to Karnovski and Roots (Karnovsky and Roots, 1964) to visualize the site of AChE activity in the cells. The very mild fixation was used to prevent the inactivation of the small forms of AChE, typical of embryonic and non-neuromuscular cell-to-cell communication (Minganti and Falugi, 1980). The slides were incubated overnight in the medium containing ferricyanide-thiocholine, with AcThCI as a substrate. After overnight incubation, the slides were dehydrated and cleared with glycerol 1:1 seawater, mounted with glycerol-gel, and observed in the optical microscope.

2.5.3. FIF reaction

Formaldehyde-induced fluorescence technique (FIF) suggesting biogenic amine expression was performed to verify the maintenance of SUCs culture excitability. SUCs on APA were incubated overnight with formaldehyde vapor, rinsed in UFSW, embedded in PEG/glycerol gel (1:4) and observed under the optical microscope.

*2.6. Expression and functionality of neuroactive molecules*

2.6.1. Biochemical assessment of AChE activity

AChE activity was measured by applying the quantitative method of Ellman et al. (Ellman *et al.*, 1961), which was modified ad hoc for the Jenway (6405 Ultra Violet/Visible, Barloworld Scientific Ltd T/As Jenway, Gransmore Green, UK) spectrophotometer. The cells cultured on APA were collected, homogenated and sonicated for 25 min in a bath sonicator (FALC, mod. LBS1, Italy). In addition, to demonstrate the maintenance of non-self-reaction ability, coelomocytes from different sea urchins cultured on APA were mixed. The cells were passed through a very thin syringe needle (Ultrafin 29G, 12.7 mm length) in the presence of 1% triton X100, and centrifuged for 30 s at 8000 rpm. The colorimetric reaction was recorded for 10 min at 412 nm wavelength in the spectrophotometer. Each measurement was performed in triplicate. A negative control consisting in coelomocytes cultured on glass slides was performed.

2.6.2. Muscarinic receptor-like immunoreactivity

To detect muscarinic receptor-like immunoreactivity for verifying excitable cell profile of culture on APA, SUCs were fixed with 3% paraformaldehyde on the APA disks and rinsed in cold PBS containing 0.5 M glycine, blocked with PBS containing 1% bovine serum albumin (BSA, Sigma-Aldrich) and 5% normal goat serum (NGS, Sigma-Aldrich). Cells were incubated overnight at 4°C with the primary monoclonal antibody M35 (Chemunex, France), raised against affinity purified calf forebrain muscarinic receptor, diluted 1:200 in PBS-0.1% BSA-1% NGS. Samples were then rinsed in PBS, and stained with the secondary fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM (Sigma) or IgG

(Sigma), diluted 1:100 in PBS containing 0.1% BSA and 1% NGS. Specificity controls were performed using normal serum as primary antibody, or by omitting the incubation in the primary antibody.

### *2.7. Statistical analysis of data*

All experiments were repeated three times. Data are expressed as means ± standard deviation of 3 replicates. Statistical analyses were conducted using SPSS statistical software version 20 (Statistical Package for the Social Sciences). Comparison of means was carried out using Student t-test and analysis of variance (ANOVA). Data were considered significantly different when $p<0.05$.

## 3. Results

### *3.1. Cell quality and viability*

The APA substrate on which the SUCs were cultured were characterized by a regular pattern of pores, with approximate pore size and pitch of 150 and 250 nm, respectively, as shown in the representative SEM image of Fig.2a.

When the APA was placed in the PLA tanks (Fig.2b), the SUCs appeared to adhere to the surface. Unstained red amoebocytes were present on the substrate after rinsing in seawater of the whole discs (Fig.2c), while DAPI and Karnovski and Roots staining allowed to detect other cell types (Fig.2d,e), such as different kind of amoebocytes and phagocytic cells (Fig.2f a-c), spherulae showing secretory granules (Fig.2f d), irregular shaped cells (Fig.2f f) and vibratiles (Fig.2f g). Cell viability was demonstrated by DAPI staining: the nuclei were intact, and all cells were characterized by the same oval shape and size (Fig.2d). DAPI and Karnovski and Roots staining show the abundance of coelomocytes and their wide distribution on the shown APA substrate (Fig.2d,e).

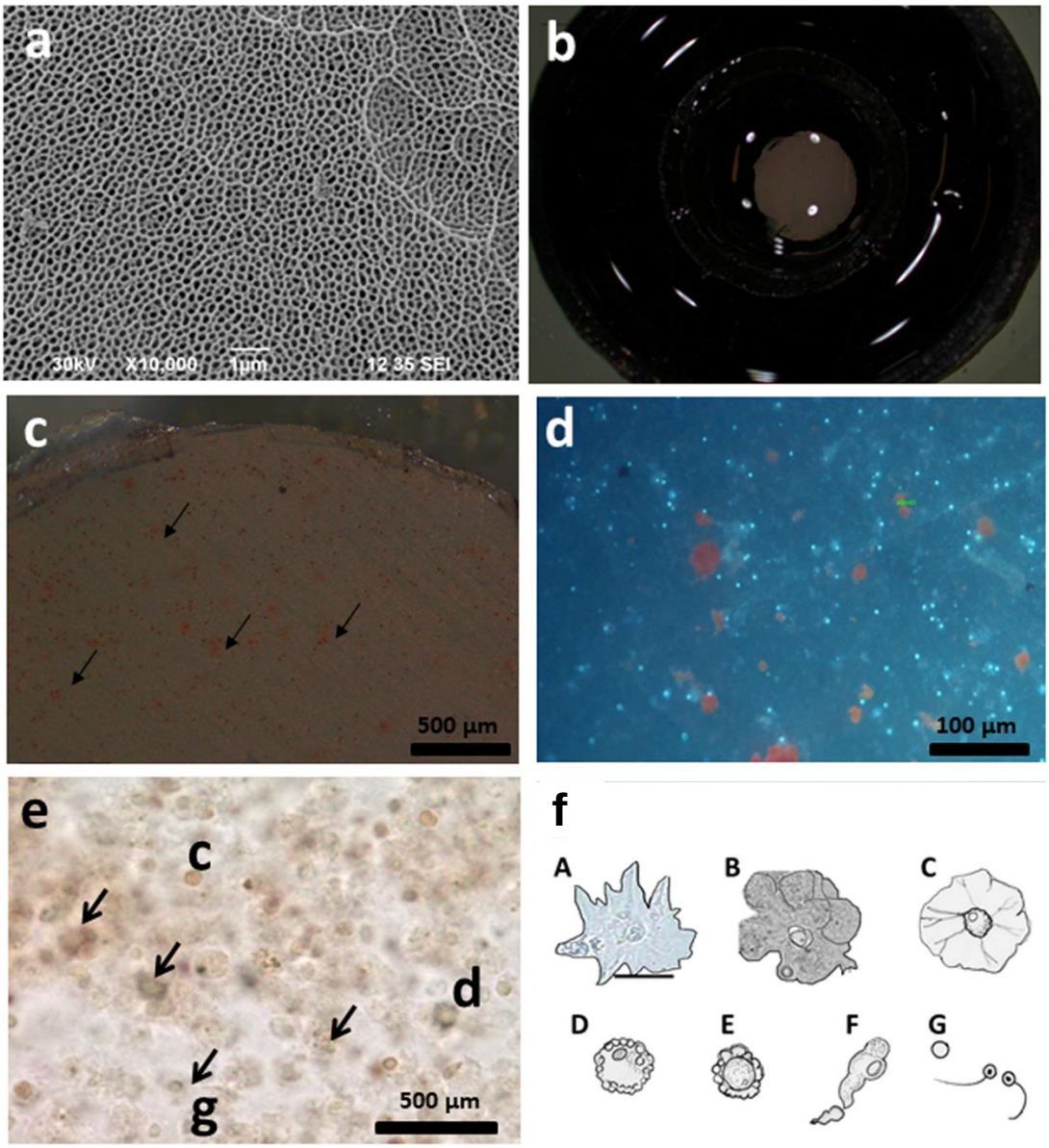


**Fig. 2** APA substrates and appearance of SUC cells cultured on them. a) APA texture; b) tank containing SUCs on APA disc on the bottom (in orange); c) SUCs (red spherulae, arrows) on the APA viewed under a stereomicroscope; d) DAPI staining showing SUCs vitality; e) Karnovski and Roots staining obtained by AChE; f) sketch of SUC types where those found in the culture can be recognized: c: phagocytic cells, d and e: spherulae showing secretory granules, g: vibratiles

*3.2. AChE activity cytochemical localization*

AChE activity shown in Fig.2e was localized in all the immune cells, mainly found in perinuclear region (site of AChE synthesis in excitable cells) and also in phagocytic cells, which are recognized by the enlarged cytoplasm (Fig.3). Nevertheless, some positive granules were present in the cytoplasm of some cells (arrows) more or less densely packed.

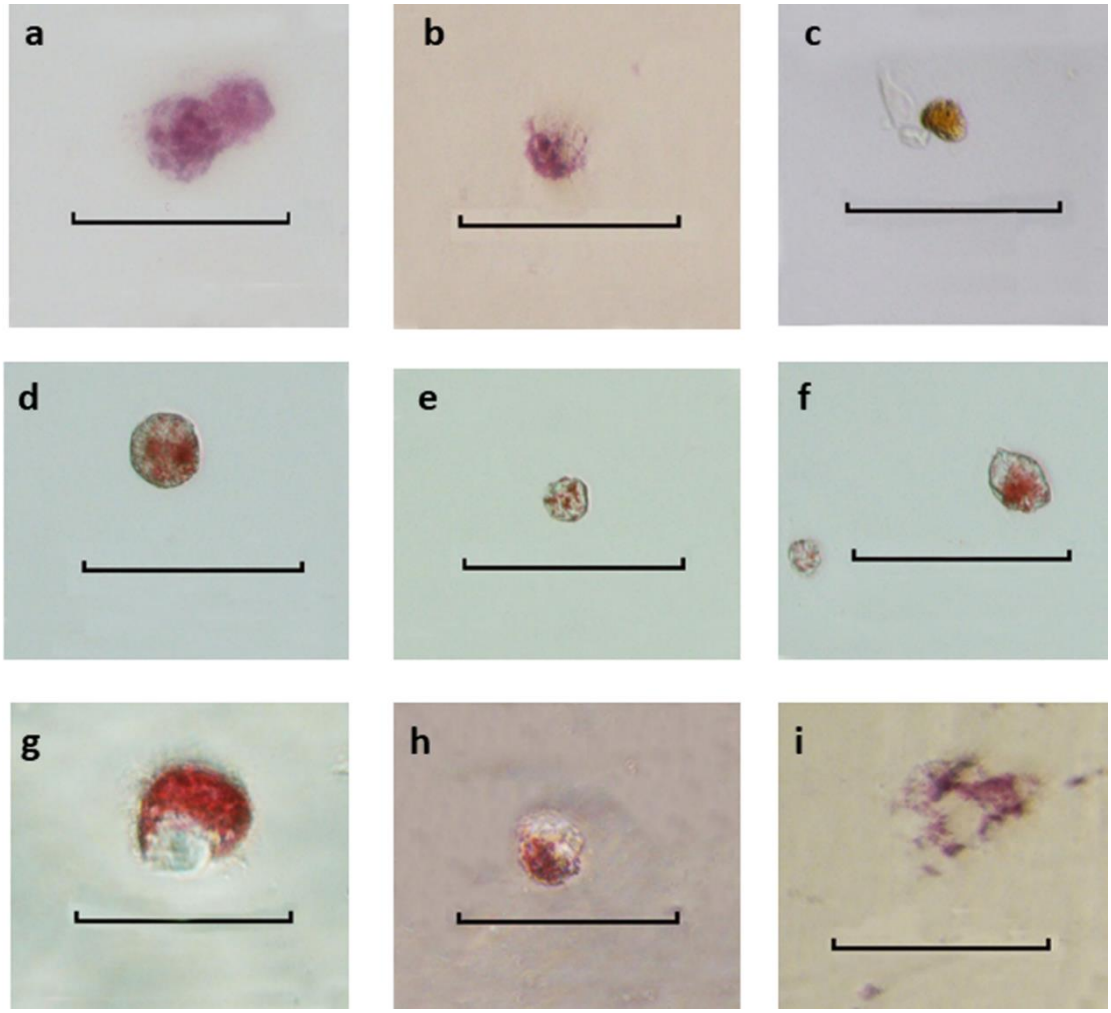


**Fig. 3** Physiological AChE response of SUCs in culture. AChE activity, revealed by the Karnovsky and Roots (Karnovsky and Roots, 1964), characterized by dark brown reaction product at the active sites. a) amoebocyte; b-d) different aspect of spherulae; e) small spherula; f) medium and small spherule; g) huge red spherule. Magenta color is due to the combination of the AChE reaction and the natural red pigment; h) medium red spherula; i) amoebocyte. Bar equals 100 µm

*3.3. FIF reaction*

FIF reaction was only found in the spherulae cells (Fig.4).

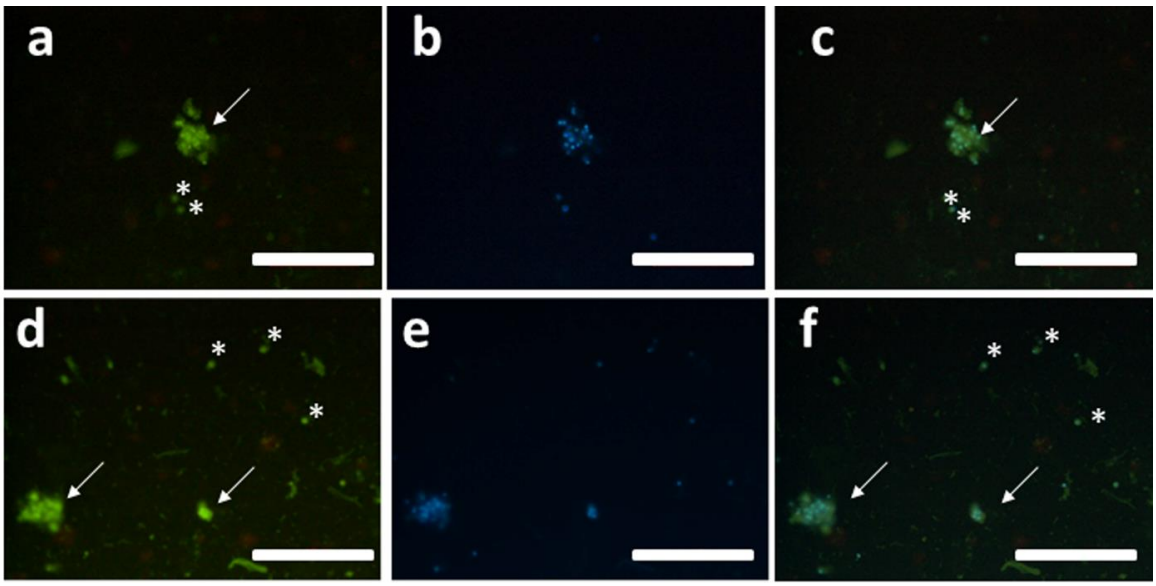


**Fig. 4** Fluorescence imaging of coelomocytes in culture. a-d) Serotonin-like fluorescence (FIF); b-e) DAPI staining of SUCs cultured on APA after 5 DIV. Single (asterisks) and cluster (arrows) SUCs showed autofluorescence induced by glutaraldehyde, confirmed by the merging of FIF and DAPI (c-f). Bar equals 100 µm

The green fluorescence (FIF) revealed the presence of serotonin-like molecules (Fig.4a, d). On the contrary, catecholamine molecules were not detected, since no co-localization of FIF and DAPI can be observed in coelomocyte cytoplasm of single cells or cluster (Fig.4c,f). A serotonin-like autofluorescence was found around the nuclei and in the cytoplasm of spherulae.

*3.4. Expression and functionality of neuroactive molecules*

3.4.1. Biochemical assessment of AChE activity

SUCs cultured for 5 DIV on APA showed the same pattern as those cultures on glass slides do, indicating that the AChE activity was not affected by APA (Fig.5).

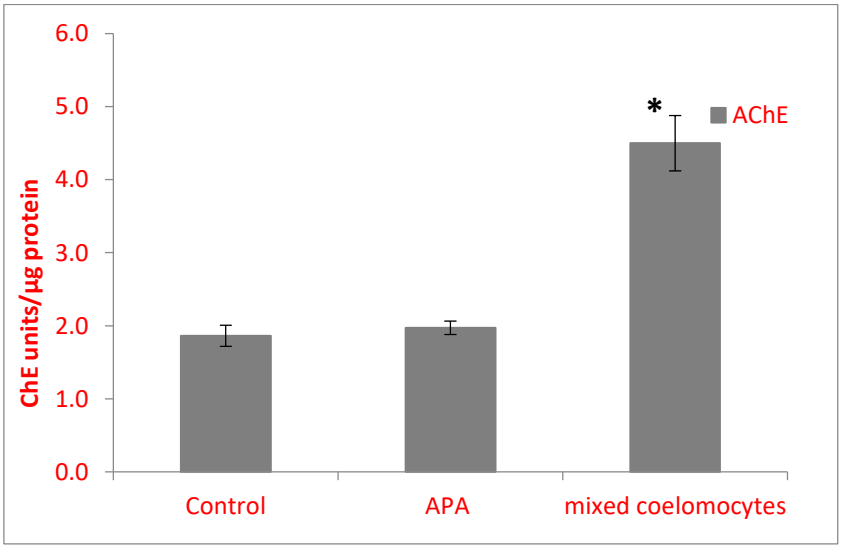

**Fig. 5** Quantitative data of AChE activity of SUCs at DIV 5. The leftmost bar shows the case of a glass slide (control) substrate, and the middle bar shows the case of APA substrate, both for cells from a single sea urchin. The rightmost bar shows AChE activity measured in mixed coelomocytes from different sea urchins. Note that mixed coelomocytes show a significant increase of AChE activity (* $p<0.05$)

Non-self-reaction performed by mixed cells from different sea urchin specimens showed a significant increase of AChE activity as compared to the single-specimen homogenates (Fig.5, * $p<0.05$).

3.4.2. Presence and localization of muscarinic receptor-like immunoreactivity

Immunofluorescence for molecules immunologically related to muscarinic receptor (M1) was localized in a subpopulation of huge cells, presenting cytoplasm elongation (Fig.6a).

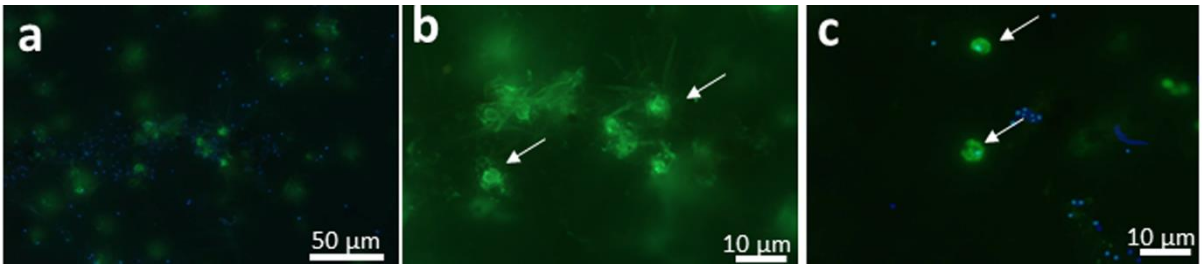


**Fig. 6** Immunoreactivity of M1- receptors in SUCs. a) overview of the mean distribution of immunoreactivity in the coelomocytes (bar equals 50 µm); b-c) M1- immunoreactivity in phagocytic cells showing cytoplasm elongation (arrows in b) and spherulae (arrows in c), (bar equals 10 µm)

The immunofluorescence was mainly present at the inner cell body, of about 5 µm diameter, with a relatively small nucleus, while the elongations were faintly stained (Fig.6b). This population represented about 1/5 of the whole number of cells, as demonstrated by DAPI staining of the nuclei in the microscope field. The size and shape of the positive cells correspond to the description of the phagocytic cells with a philopodial shape (Pinsino and Matranga, 2015). Fig.6c shows the Muscarinic-like immunoreactivity in cells previously described by Smith *et al* (Smith *et al.*, 2010) as spherulae cells and later on as white amoebocytes (Smith *et al.*, 2018). Also in his case, the cell body is positive, with a central-lateral nucleus. The other cells shown by DAPI staining are not positive.

## 4. Discussion

In this study the possibility to culture SUCs on APA biocompatible substrate for marine environmental monitoring has been demonstrated. To the best of our knowledge, so far SUCs used in biological studies have been cultured mainly on standard culture surfaces, such as Petri dishes, glass slides or other smooth substrates, same as for the case of the studies already reported and commented herein. Generally speaking, for all types of living cells, adhesion and proliferation has proven to be easier and more robust on a nanoscale rough substrate, rather than on a smooth one. Additionally, the nanoporous nature of the APA roughness is open for *in vivo* delivery of bioactive agents possibly loaded in the pore before seeding, allowing to a kind of surface bio-functionalization, where necessary.

Despite the SUCs cultured on APA presented signals of sufferance, living cells were observed up to 5 DIV, indicating that APA ensures cell adhesion. Similar results were observed for a variety of human cells cultured on APA (Salerno *et al.*, 2013; Toccafondi *et al.*, 2014). The APA structure and in particular the nanoroughness of its pores seems to provide pinning points to the cell membrane, triggering focal adhesions of similar nanoscale size (El Merhie *et al.*, 2019).

It should be noticed as a limitation of the present study that some cells in Fig.2d, 4 and 6 appear with diffuse scattering background or out of focus. This is partly due to the nature of APA as a rough and even wavy or curved surfaces. In fact, the consumer foil was easily bent and deformed in shape during manipulation, which resulted in a non-planar surface, different from glass or silicon wafer supported APA surfaces (C. Toccafondi *et al.*, 2016; El Merhie *et al.*, 2019).

The immune cells function as the central sensing and effector components of *P. lividus* sea urchin reside within the coelomic cavity, guaranteeing key functions, from chemotaxis to cytotoxic responses (Pinsino and Alijagic, 2019). The main morphologically and likely functionally distinct circulating immune cell types (phagocytes, amoebocytes, vibratile cells) were found upon APA substrate after 5 DIV. These cells colonize the coelomic fluid of the sea urchins in similar way as the leucocytes of the blood of higher animals do (Pinsino and Matranga, 2015). This biological fluid contains essential trophic and activating molecules (proteins, metabolites) produced by immune cells to direct their own behavior (Alijagic and Pinsino, 2017). The latter was not strongly affected in the APA cultured cells, since cholinesterase and serotonergic activity, the expression and functionality of neuroactive molecules (AChE, M1 acetylcholine receptors) were not impaired after 5 DIV culture. In this regard, AChE activity was located mainly around the nuclei of the sea urchin immune cells, according to previous studies where these cells were cultured on other materials, such as glass slides (Angelini *et al.*, 2003). This localization corresponds to the site of synthesis of AChE molecules in neuromuscular and non-neuromuscular excitable cells, where the activation of acetylcholine receptors (both muscarinic and nicotinic) occurs. AChE activity measured in immune cells cultured on APA did not differ from that found in other substrates (i.e. glass), conversely to non-self-reaction, performed by mixing cells from different sea urchin specimens. The latter showed an increase of enzyme activity, as compared to single specimens, indicating an inflammatory response as reported in human immune cells (Wessler and Kirkpatrick, 2008).

SUCs cultured on APA maintained the biochemical behavior of excitable cells, as revealed by serotonergic activity (in terms of serotonin-like auto fluorescence), and the expression and functionality of neuroactive molecules. The nature of excitable cells for sea urchin was previously demonstrated, as well as other features, such as the presence of nicotinic AChE receptors in the cytoplasm of spherulae and the biosynthetic enzyme of acetylcholine (namely choline acetyltranspherase) in phagocyte granules and in spherulae cytoplasm (Gambardella *et al.*, 2015). These results, together with the presence of muscarinic receptors in huge phagocytic cells and serotonin in the spherulae cultured on APA, show that SUCs may respond to environmental (intracellular and intercellular) stress through ionic dynamics (Anselmo and Berg, 2012; Falugi and Aluigi, 2012). In this regard, serotonin modulates calcium influx in neural cells (Diaz-Rios *et al.*, 2019), while muscarinic receptors activate cascade in the cytoplasmic domain, causing calcium mobilization from the intracellular stores (Felder *et al.*, 1995). On this basis, the excitable cell profile is maintained after 5 DIV culture on APA, since serotonin-like auto fluorescence and M1 immunoreactivity was found in the main immune cell types.

These findings are also supported by histochemical and biochemical AChE activity, which suggest that electrical events are not lost after culture on APA. Serotonergic, cholinergic and muscarinic systems are involved in the electrical events: since they were not impaired, the APA substrate can potentially be a good material for biosensor development based on electrical readout. The development of electrical APA-based SUC biosensors could be possible, using the aluminium remained in place under the top oxide layer resulting from anodization as an electrode for either input or output, monitoring DC or AC impedance, and carrying out barrier-layer thinning according to established protocols (Stępniowski *et al.*, 2015). The latter process allows to open the pore bottoms, making the medium above the APA to contact directly the underlying electrode (Furneaux, Rigby and Davidson, 1989): this improvement should be considered for biosensor validation in field. Indeed, Graham et al have already demonstrated electrical connection of sensitive neuronal cells, cultured on APA (Graham *et al.*, 2009, 2010). In this way, a final SUC device able to monitor online different target contaminants such as nanoparticles and pesticides, known to affect the SUC's cholinergic system, could be easily developed and tested in field.

In our laboratory tests, PLA was the material chosen for the SUCs culture tanks, since it is biodegradable and it can be obtained from renewable resources. PLA is among the most commonly used materials for the construction of objects through 3D printers, since it is easy to work (no hot plate is requested), no special precautions are necessary, and no toxic vapors are produced during processing. Moreover, PLA can be degraded in lactic acid and is used as medical implants, so it can be considered completely biocompatible. Regarding the shape of the tanks, the used prototype has already been designed with the aim of ensuring uniformity of the measure. This means that the cylindrical shape allows the diffusion of the pollutant in axial symmetry towards the sensor (see Fig.2b), so that the response of the cells to the stressor is a variable dependent on a single spatial coordinate, namely the radius of the support. In this way, it could be possible to use semi-analytical solutions of the diffusion advection equation to study the time response of the coelomocytes to the pollutants. The PLA tanks have been proven to guarantee oxygen exchange, required for SUCs survival up to 5 DIV. As with PLA different textures can be easily realized, in the future it may be

interesting to evaluate the possible construction of tanks in which the diffusion of oxygen through the walls is allowed, and the fabrication of textures specifically designed to select the passage or not of different pollutant molecules. Obviously, given the high biodegradability of PLA, it would not be the best material to be subjected to sterilization techniques using heat and/or UV and to be put in direct and prolonged contact with seawater. However, considering the single-use for which the tanks will be designed, the expected duration, even with tanks subjected to the sterilization procedure, is more than sufficient. We will proceed with the implementation of an experimental campaign to evaluate the effective duration of unaltered mechanical and chemical-physical properties of the PLA tanks if subjected to different sterilization techniques (microwave, UV) and immersed in seawater for the time considered necessary for the validity of the possible biosensor, which could be one week.

At present, a number of different techniques are available for monitoring the marine environment (Mills and Fones, 2012; Danovaro *et al.*, 2016; Bean *et al.*, 2017). In addition to requirements for low cost and easy disposal of used materials (Gille *et al.*, 2014), one of the key issues is the time of response, which can vary from the scale of several months, for traditional taxonomic analysis of the collected sediment samples, down to few weeks only, when using genomic tools (7th Framework Programme - European Commission Project, 2016). However, collection and on-place analysis based on simple electrical readout - possibly combined with optical and/or fluorescence imaging - of disposable devices set at monitoring sites, could provide response in one day, limiting the time to mostly the device collection step. Using biosensors based on living marine cells (Justino *et al.*, 2015; 7th Framework Programme - European Commission Project, 2018) would probably make readout faster and easier, certainly less selective yet more universal in the broad range of adverse polluting effects possibly identified (Kröger, Piletsky and Turner, 2002). The APA nanopores have been already proposed for electrical sensing in general applications (de la Escosura-Muñiz and Merkoçi, 2016). We suggest here to take one more step in a specific field of application, namely the marine water monitoring by means of SUCs.

## 5. Conclusion

This study demonstrates that SUCs can be cultured on APA substrates, which is the first step in moving towards the use of these cells as the core of a biosensor. Besides ensuring cell adhesion, it is shown here that the APA does not affect the biochemical behavior and the cholinergic system pathway of the SUCs, indicating that homeostasis and intercellular crosstalk were maintained, thus suggesting its potential to be proposed for marine environment biosensing, after system refinement. Our findings provide a possible approach to construct an electrical sensitive tool for measuring immune responses of SUCs to be further used for marine environmental assessment. The PLA tanks seem promising as the case for the biosensing chamber, for both stability, biocompatibility, and ease of manufacturing. Additionally, together with biocompatible APA, future fabrication of the biosensor from these materials would also guarantee safe final disposal of the used biosensor devices in the environment, after their service. Further work will address the patterning of the nanoporous regions and electrical connection of the defined pads.

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**Ethical approval**

All procedures performed in this study involving animals (sea urchin *Paracentrotus lividus*) were in accordance with European ethical standards. The type of animals involved in this research are included in the Annex III of the Marine Strategy Framework Directive 2008/56/EC on the approximation of laws, regulation and administrative provisions regarding the protection of animals used for experimental and other scientific purposes. The regulation lays down what is commonly known as the 3Rs (Reduce, Refine and Replace) approach.

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Captions:

**Fig. 1** Sketch of the tanks used for cell incubation to optimize diffusion. a) 3D tank overview; b) section of the tank with diffusion path (arrows) and description of loading media and accessory components

**Fig. 2** APA substrates and appearance of SUC cells cultured on them. a) APA texture; b) tank containing SUC on APA disc on the bottom (in orange); c) SUCs (red spherulae, arrows) on the APA viewed under a stereomicroscope; d) DAPI staining showing SUCs vitality; e) Karnovski and Roots staining obtained by AChE; f) sketch of SUC types where those found in the culture can be recognized: c: phagocytic cells, d and e: spherulae showing secretory granules, g: vibratiles

**Fig. 3** Physiological AChE response of SUCs in culture. AChE activity, revealed by the Karnovsky and Roots (Karnovsky and Roots, 1964), characterized by dark brown reaction product at the active sites. a) amoebocyte; b-d) different aspect of spherulae; e) small spherula; f) medium and small spherule; g) huge red spherule. Magenta color is due to the combination of the AChE reaction and the natural red pigment; h) medium red spherula; i) amoebocyte. Bar equals 100 µm

**Fig. 4** Fluorescence imaging of coelomocytes in culture. a-d) Serotonin-like fluorescence (FIF); b-e) DAPI staining of SUCs cultured on APA after 5 DIV. Single (asterisks) and cluster (arrows) SUCs showed autofluorescence induced by glutaraldehyde, confirmed by the merging of FIF and DAPI (c-f). Bar equals 100 µm

**Fig. 5** Quantitative data of AChE activity of SUCs at DIV 5. The leftmost bar shows the case of a glass slide (control) substrate, and the middle bar shows the case of APA substrate, both for cells from a single sea urchin. The rightmost bar shows AChE activity measured in mixed coelomocytes from different sea urchins. Note that mixed coelomocytes show a significant increase of AChE activity (* $p<0.05$).

**Fig. 6** Immunoreactivity of M1- receptors in SUCs. a) overview of the mean distribution of immunoreactivity in the coelomocytes (bar equals 50 µm); b-c) M1- immunoreactivity in phagocytic cells showing cytoplasm elongation (arrows in b) and spherulae (arrows in c), (bar equals 10 µm)