Mineral-bearing vesicle transport in sea urchin embryos

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Key words

Biom mineralization, Filopodia, Sea urchin spicule, Vesicle transport, cryo-FIB-SEM, Raman spectroscopy

Abstract

Sea urchin embryos sequester calcium from the sea water. This calcium is deposited in a concentrated form in granule bearing vesicles both in the epithelium and in mesenchymal cells. Here we use *in vivo* calcein labeling and confocal Raman spectroscopy, as well as cryo-FIB-SEM 3D structural reconstructions, to investigate the processes occurring in the internal cavity of the embryo, the blastocoel. We demonstrate that calcein stained granules are also present in the filopodial network within the blastocoel. Simultaneous fluorescence imaging and Raman spectroscopy show that these granules do contain a calcium mineral. By tracking the movements of these granules, we show that the granules in the epithelium and primary mesenchymal cells barely move, but those in the filopodial network move long distances. We could however not detect any unidirectional movement of the filopodial granules. We also show the presence of mineral containing multivesicular vesicles that also move in the filopodial network. We conclude that the filopodial network is an integral part of the mineral transport process, and possibly also for sequestering calcium and other ions. Although much of the sequestered calcium is deposited in the mineralized skeleton, a significant amount is used for other purposes, and this may be temporarily stored in these membrane-delineated intracellular deposits.

Introduction

In less than three days after fertilization sea urchin embryos develop a calcium carbonate skeleton consisting of two spicules (Millonig, 1970; Okazaki and Inoue, 1976; Politi et al., 2006; Wilt and Benson, 1988). This rapid mineralization process involves massive sequestering and transport of calcium and carbonate ions to the forming spicule. The calcium ions are sequestered from sea water, whereas the carbonate ions are both sequestered from sea water and are the product of metabolic processes (Sikes et al., 1981). Calcium enters into the embryonic body cavity (blastocoel), probably in the form of ions in solution or possibly nano-sized clusters (Decker and Lennarz, 1988; Hwang and Lennarz, 1993; Nakano et al., 1963; Stumpp et al., 2012; Tellis et al., 2013). The first appearance of calcium carbonate in a concentrated form is observed inside both epithelial cells and primary mesenchyme cells (PMCs) (Beniash et al., 1999; Decker and Lennarz, 1988; Hwang and Lennarz, 1993; Okazaki, 1975; Vidavsky et al., 2014; Wilt et al., 2008). These calcium carbonate deposits consist of nanospheres 20-30 nm in diameter, and are located in an intracellular environment inside membrane delineated vesicles (Beniash et al., 1999; Vidavsky et al., 2014). The blastocoel separates the epithelial tissue from the tissue containing the PMCs. The focus of this study is on the blastocoel, in particular on the dense network of filopodia that connects the epithelium with the PMCs. We use *in vivo* calcein labeling to track calcium deposits and movements, *in vivo* Raman spectroscopy to characterize the mineral deposits and the novel method of cryo-FIB-SEM to better understand 3D aspects of this filopodial network preserved in a high pressure frozen embryo.

Filopodia are thin cytoplasmic extensions of cells that are composed of actin filaments covered by a plasma membrane. They are flexible and strong, and in the sea urchin embryo they grow and retract at rates of up to 10 $\mu\text{m}/\text{min}$ (Jacinto and Wolpert, 2001). PMCs, epithelial cells and secondary mesenchyme cells (SMCs) in the sea urchin embryo extend thick ($\sim 1\mu\text{m}$ in diameter) and thin (0.2-0.4 μm in diameter) filopodia. These filopodia can extend to lengths of tens of microns and are involved in cell-cell interactions associated with cell movement (thick filopodia), signaling and skeletogenesis patterning (thin filopodia) (Gustafson and Wolpert, 1963; McClay, 1999; Miller et al., 1995). It is known that PMC migration and spicule shape are determined through PMC filopodia by VEGF (Vascular Endothelial Growth Factor) signaling from the epithelial cells (Duloquin et al., 2007; Knapp et al., 2012), as well as through

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4 interactions with the extracellular matrix (Hodor et al., 2000). PMCs and SMCs come into direct
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6 filopodial contact during the transmission of cell fate signaling (Ettensohn, 1992). Filopodial
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8 connections between mesenchymal and epithelial cells were hypothesized to be involved not
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10 only in cell-cell signaling, but also in nutrient transport (Stumpp et al., 2012). Most significantly
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12 for this study, ‘calcareous granules’ were observed within the filopodial strands by light
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14 microscopy and electron dense granules were detected by TEM (Decker and Lennarz, 1988;
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16 Okazaki, 1960). Here we investigate the dynamic movement of calcium carbonate granules in
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18 the filopodial network, and produce high resolution 3D reconstructions of the blastocoel
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20 environment and of the vesicles incorporated within the filopodia.

21 22 **Materials and Methods**

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24 **Sea urchin embryonic/ larval culture.** Mature cultured adult sea urchins *Paracentrotus lividus*
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26 were produced and supplied by the Israel Oceanographic and Limnological Research Institute.
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28 Spawning was induced by injecting 1 mL of 1M KCl solution into the coelomic cavity. The egg
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30 suspension was collected, washed, and kept in sea water containing 30 mg/L Penicillin G sodium
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32 salt (Sigma–Aldrich, 69578) and 15 mg/L Streptomycin sulfate salt (Sigma-Aldrich, 3810740) at
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34 18 °C. Sperm was kept at 4 °C undiluted. The fertilization was carried out by sperm dilution in
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36 sea water and rapid mixing with the egg suspension inside sea water containing 30 mg/L
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38 Penicillin G sodium salt (Sigma–Aldrich, 69578) and 15 mg/L Streptomycin sulfate salt (Sigma-
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40 Aldrich, 3810740). The time of mixing is defined as time 0 post fertilization. The culture was
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42 kept at 18 °C with gentle shaking (100 rpm). The developmental stage of the embryo was
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44 determined from the spicule and embryo characteristic morphology, using a light microscope
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46 with normal and polarized light. When the desired prism or pluteus (Giovanni, 1973)
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48 developmental stage was reached, which occurs approximately 42-49 h post fertilization, the
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50 samples were imaged *in vivo* or high pressure frozen without any processing, as described below.
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52 The research involving sea urchins is approved by the Israel Oceanographic and Limnological
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54 Research, National Center for Mariculture.

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56 **Calcein labeling.** Calcein, 20 μ M (Sigma–Aldrich, 154071484), was dissolved in sea water
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58 containing 30 mg/L Penicillin G sodium salt (Sigma–Aldrich, 69578) and 15 mg/L Streptomycin
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60 sulfate salt (Sigma-Aldrich, 3810740) and filtered in a 0.22- μ m sterile Corning filter system. For
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continuous labeling, developing embryos were immersed from time 0 in calcein-labeled sea water at 18 °C with gentle shaking (100 rpm). At the prism or pluteus stage, the embryos were washed with sea water and examined. For pulse labeling, the embryos were transferred to 20 μ M calcein in sea water for 45 minutes at the prism or pluteus stage, or at 26h post fertilization and until the pluteus stage was reached. After the labeling, the embryos continued to grow in unlabeled sea water.

Sample mounting for microscopy and spectroscopy experiments. 1 μ L of the embryo suspension was put between a glass slide and a glass coverslip, using Vaseline as a spacer to confine the embryos spatially in a hydrated environment.

Fluorescence microscopy and image processing. The fluorescence images and movies were obtained using a Deltavision system (Applied Precision) with X100 NA 1.4 and X60 NA 1.42 oil immersed objectives and confocal spinning disc system (Zeiss Observer Z1 spinning disk Yokogawa CSU-x1) with X63 NA 1.4 oil immersed objective. In the Deltavision system, calcein was excited at 490nm and the emission was detected at 525nm. In the confocal spinning disc, calcein was excited at 494nm and the emission was collected at 514nm. For Fig. 7A, 20 time points, at 10s intervals are shown. For Fig. 7B, 81 time points, at 15s intervals are shown. Images were taken with a photometrics cool SNAP HQ2 CCD from Roper Scientific. Image analysis and processing was conducted using Image J (US National Institutes of Health, Bethesda, Maryland, USA) and Adobe Photoshop CS 4 Extended (Adobe systems, San Jose, CA). Particle tracking was conducted using the MTrackJ program (Meijering et al., 2012) in 4D.

Correlative fluorescence imaging and Raman spectroscopy. The Raman setup is based on a commercially available confocal Raman microscope (Alpha 300, WITec, Ulm, Germany) equipped with a Helium Neon (HeNe) laser (633 nm) for excitation and a piezoscanner (P-500, Physik Instrumente, Karlsruhe, Germany). The spectra were acquired with a thermoelectrically cooled CCD detector (DU401A-BV, Andor, UK) placed behind a grating (600 g mm⁻¹) spectrograph (UHTS 300; WITec, Ulm, Germany) with a spectral resolution of 3 cm⁻¹. The red laser beam (633 nm) was focused through a 100x oil immersion (Nikon, NA=0.9) microscope objective. A pinhole size of 50 μ m was used. The ScanCtrlSpectroscopyPlus software (version 1.38, Witec) was used for measurement and WITec Project Plus (version 2.10, Witec) for spectra processing. The in-house implementation of the fluorescence modality on the above mentioned

confocal Raman microscope relies on the addition of two dichroic mirrors (Thorlabs, USA) and a CCD camera (Infinity3-1URM, Lumenera Corporation, Canada) in the imaging arm of the microscope, as well as two LEDs for dual excitation at 480 and 380 nm in the illumination arm. The dichroic mirrors were carefully chosen so as to not hinder imaging using Raman microspectroscopy at 633 nm. Details of a similar setup can be found elsewhere (Bennet et al., 2014).

Sample preparation for cryo imaging. Embryos and larvae at the prism or pluteus stage were high-pressure frozen: 10 μ L of the embryo suspension were sandwiched between two metal discs (3-mm diameter, 0.1 mm and 0.05 mm cavities) and cryo-immobilized in a high pressure freezing device (HPM10; Bal-Tec).

Cryo-FIB-SEM 3D imaging (serial FIB milling and block face SEM imaging under cryogenic conditions) is a recently developed cutting edge technique for high resolution 3D visualization of unstained and unprocessed samples (Schertel et al., 2013). The samples are first high-pressure frozen omitting any fixation or staining and then these native frozen samples are processed in a FIB-SEM microscope under cryogenic conditions. Thin (down to 10 nm) slices of material are removed in a serial manner by FIB milling and then each newly exposed surface is imaged by SEM using InLens secondary electron (SE) detection. Water-rich areas such as cytoplasm or extracellular space show a brighter grey level in the InLens SE image than lipid-rich structures such as membranes. The image contrast is most likely obtained due to local differences of surface potential as discussed in (Schertel et al., 2013). Cryo-FIB-SEM of a native high-pressure-frozen sea urchin embryo at the prism stage was carried out using an Auriga 60 FIB-SEM (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Throughout the imaging the samples were kept below -145°C . In a serial manner slices of the sample were removed by FIB milling using a FIB probe current of 600pA and each of the freshly exposed cross-sections was observed by SEM Imaging using InLens SE detection. Voxel sizes (image pixel size in X-direction x image pixel size in Y-direction x slice thickness) used in this study were 12.5nm x 12.5nm x 25nm at 1.45 kV (Fig. 4, 6) and 10nm x 10nm x 20nm at 1.8 kV (Fig. 3). Image processing was carried out using Image J (US National Institutes of Health, Bethesda, Maryland, USA) through FFT based filtering. Slice alignment and 3D reconstruction of the spicule environment based on the cryo-FIB-SEM images was carried out using Image J and Avizo 8 manual segmentation.

Cryo-SEM imaging. The frozen samples were kept in liquid nitrogen and transferred using a vacuum cryo transfer device (VCT 100; Leica Microsystems, Vienna, Austria) to a freeze fracture device (BAF 60; Leica Microsystems, Vienna, Austria). Samples were freeze fractured at -120°C , and etched for 10 min at -105°C . The uncoated samples were observed at -120°C in an Ultra 55 SEM (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) using an SE InLens detector and an InLens energy selective backscattered electron (EsB) detector at acceleration potentials between 1.5 and 5 kV.

Results

Sea urchin embryos were grown in calcein-labeled sea water for different time periods, rinsed with sea water at the prism or pluteus stage, and mounted between a glass slide and a cover slip using spacers that immobilize the live embryos. Light microscopic *in vivo* imaging of sea urchin embryos and larvae using transmitted and fluorescent light was performed using the Deltavision microscope or the confocal spinning disc microscope. The advantages of the Deltavision system are the high XY resolution and the high quality of the transmitted light images, and the advantages of the spinning disc confocal microscope are the high resolution fluorescence signal and rapid scanning.

Calcein labeled granules associated with the filopodial network

Using the Deltavision system, we observed a branched filopodial network of cellular extensions connecting different cells and cell types (Fig 1A). The diameters of the filopodial extensions are a few hundred nm, but vary between locations. Micron-size granules are observed along the filopodial extensions (Fig 1A). These granules are labeled with calcein (Fig 1B, Fig. 2A-B), implying the presence of a concentrated calcium-containing phase within the filopodial granules. This is in agreement with earlier observations (Decker and Lennarz, 1988; Okazaki, 1960). Stumpp et al. (2012) reported that the entire filopodial network is enveloped by membranes, including the multiple bodies and vesicles incorporated within the filopodia. The contents of the calcein-labeled granules were characterized by Raman spectroscopy using a tailor-made microscope that enables the fluorescence and Raman signals to be obtained from the same pixel (Akiva et al., 2015; Bennet et al., 2014). In this way we are certain that the Raman

spectrum is derived from the fluorescent granules. Figure 2C is the fluorescence emission image acquired using the correlative fluorescence-Raman microscope, showing the calcein-labeled spicule and a micron-size calcein labeled granule. The latter has a diameter of $\sim 2\mu\text{m}$ and was detected at a distance greater than $4\mu\text{m}$ from the spicule. Considering that the spatial resolution of the technique is $\sim 1\mu\text{m}$, this ensures that the acquired spectra were not derived from the spicule. A 2D Raman-spectrum map was acquired at the same location (Fig. 2D) with a pixel size of 500nm. The spicule is clearly identified by its Raman spectrum, showing characteristic calcite shifts (Fig. 2D, E, blue) at 283, 720, 1090 cm^{-1} of rotational modes, in-plane bending (ν_4) and symmetric stretching modes (ν_1) respectively (Unvros et al., 1991). The same spectrum of calcite was obtained from the micron-size calcein-labeled granule next to the spicule, and from 6 other granules in the tissue from other locations. Previous studies using cryo-TEM did not detect calcite in these tissues, but amorphous calcium carbonate (ACC) (Beniash et al., 1999). We therefore assume that ACC in the calcein-labeled granules was induced to crystallize by the Raman laser beam.

We next examined the blastocoel area at higher resolution paying specific attention to the filopodial network. We used cryo-SEM, as it enables preservation and imaging of biological samples in vitrified water without any staining or chemical processing, and thus minimizes the risk of losing labile mineral phases such as ACC. Figures 3A and B show the proximity of tube-like structures, which based on shape and diameter are attributed to filopodia. These are located next to the membrane-bound forming spicule. We thus conclude that the filopodial network extends into the area immediately surrounding the forming spicule.

We used cryo-FIB-SEM 3D imaging in order to obtain a high resolution 3D reconstruction of the filopodial network and the associated vesicles incorporated within the filopodia. This novel technique allows ultrastructural imaging in all three dimensions by iterative slicing and imaging of the native high pressure frozen embryo omitting any staining or chemical fixation at a voxel size down to around 10 nm and below (Schertel et al., 2013) (see methods). The blastocoel space is surrounded by epithelial cells and primary mesenchymal cells, and is densely populated by vesicles with diameters of 0.5-1 μm (Fig 3C, 4A). Smaller features with diameters of around 100 nm are attributed to cross-sections of filopodia (Fig 4A). These features can be followed in sequential images. Occasionally filopodia can be seen in longitudinal section, when they lie in the plane of the image. When this occurs, granules are observed along the filopodia (Fig. 4B).

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4 The cryo-FIB-SEM 3D reconstruction unequivocally shows that the filopodia extending into the
5 blastocoel are far from epithelial cell bodies or PMC cell bodies and contain distinct granules of
6 the same size as those observed with fluorescence microscopy.
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10 While monitoring the calcein-labeled vesicles inside the filopodia we noticed that some clusters
11 of vesicles are present inside membrane-enveloped bodies. These vesicle clusters are a few
12 micrometers in diameter, and are hence much larger than the more abundant sub-micron sized
13 individual vesicles. They are also located along the filopodial network (Fig. 5A, B) and move
14 along it in the same manner as the smaller individual vesicles. Correlative *in vivo* fluorescence-
15 Raman of these large multivesicular vesicles, shows the characteristic carbonate Raman shifts of
16 calcite (286, 720, 1090 cm^{-1}) (Fig 5 C, D), demonstrating that they do contain the mineral calcite.
17 As noted above, the detection of crystalline calcite is most likely due to beam-induced
18 crystallization of a calcium carbonate phase. In this case we noted that the same particles display
19 no crystallinity under polarized light. Cryo-SEM micrographs of high pressure frozen and freeze
20 fractured embryos also show the existence of multivesicular vesicles containing within them
21 smaller vesicles that in some cases are embedded in cell cytoplasm (Fig. 6A, B). Using the cryo-
22 FIB-SEM for obtaining high resolution (25nm slice thickness) 3D images, we verified in the
23 sequential sections that these multivesicular vesicles contain no cellular nucleus (Fig 6 C-H,
24 Video SI5). Furthermore, the individual vesicles have different textures that may be indicative of
25 different compositions (Fig. 6D-H). Note that membrane lipids typically appear dark in these
26 images, whereas different components of the multivesicular vesicles appear in varying shades of
27 gray. Since the multivesicular vesicles are a few micrometers in diameter and are calcein-
28 labeled, it is probable that the calcein-labeled bodies analyzed by Raman spectroscopy are the
29 same as the vesicles observed here.
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50 Movement of the calcein labeled granules

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52 We monitored the movement of the calcein labeled granules using fluorescence light microscopy
53 in the time lapse mode. Figures 7 A, B and videos SI1-3 show the tracks of the calcein-labeled
54 granules. All the calcein-labeled granules detected inside epithelial cells and almost all the
55 granules observed inside PMCs hardly moved. In fact they did not leave the cells as granules. In
56 contrast, almost all the granules located inside filopodia traveled distances longer than 5 μm ,
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4 indicating that the filopodia play a major role in granule transport within the embryo. Figure 7A
5 is an example of the various granule pathways at different locations. We did not observe any
6 unidirectional transport of granule-bearing vesicles towards, for example, the forming spicule or
7 away from it. A summary of the calcein-labeled granule movements per location is shown in
8 Table 1.
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14 **Table 1.** Calcein-labeled granule movement per location in the sea urchin embryo. The data were
15 collected from videos SI 1-4, based on a total of 105 particles.
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Granule location	Number of granules observed in location	Percentage of granules observed moving a long distance (>5µm) per location
Epithelial cells	28	0%
Filopodia	53 (17 in vicinity of the spicule)	94%
PMCs	24	8%

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35 **Discussion**
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37 Here we show that mineral-bearing vesicles are found not only in epithelial cells and primary
38 mesenchyme cells, but also in the intracellular environment of filopodial extensions located in
39 the blastocoel (body cavity). This confirms earlier observations (Decker and Lennarz, 1988;
40 Okazaki, 1960). We observed two different modes of vesicle movement: the mineral-bearing
41 vesicles in epithelial cells and in PMCs remain within the cells during the time of observation
42 and move very short distances, whereas vesicles inside filopodia travel relatively long distances.
43 The mineral-bearing vesicles in the filopodial network did not move in one clear-cut direction.
44 We also identified mineral-bearing multivesicular vesicles moving within the filopodial network.
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53 Vesicles inside epithelial cells moved very short distances and remained within the cells (at least
54 on the time scale of our observations). It is therefore possible that the epithelial calcium-bearing
55 vesicles function as a solid state calcium reservoir needed for a variety of purposes, including for
56 signaling events during embryonic pattern formation, cell differentiation, cell proliferation and
57 apoptosis (Berridge et al., 2000; Whitaker and Patel, 1990). As embryos at this stage are
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4 swimming vigorously via cilia and the regulatory mechanism of ciliary activity is sensitive to
5 calcium concentration (Degawa et al., 1986), the calcium reserves may also be used for
6 regulating the beating of cilia. We note that in cellular signaling processes, it is necessary to
7 maintain high amounts of calcium isolated from the cytoplasm, and hence packaging of
8 concentrated ion deposits inside a vesicle would serve this purpose. Organelles that are known to
9 store calcium are the endoplasmic reticulum (ER) and the mitochondria (Clapham, 1995), but
10 other calcium storage structures include acidocalcisomes, vacuoles, lysosome-related organelles
11 and vesicles, are known (Patel and Docampo, 2010). It is also possible that the mineral particles
12 observed in the epithelial cells are storage sites for future mineralization events, such as for the
13 later development of the embryo and when it forms test, teeth, and spines. We note too that a
14 role for calcium other than skeletal building is consistent with the observation using ^{45}Ca that
15 only 70% of the calcium taken up by the embryo is deposited in the spicule, and 30% is utilized
16 for other purposes (Fujino et al., 1985; Mintz et al., 1981).

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18 The filopodia extend for lengths of up to tens of microns from the cell body into the blastocoel.
19 They connect the PMCs to each other, and are located in close proximity to the forming spicule.
20 The PMC filopodia have contact points with epithelial cells that also extend filopodia into the
21 blastocoel. The filopodial complex spatial structure allows vesicle transport between different
22 epithelial cells and the primary mesenchymal cells, including vesicles that contain mineral. One
23 key question is what is the source of the ions that form these filopodial associated minerals?

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25 Experiments in which embryos were grown in either ^{45}Ca or calcein in sea water, show that the
26 initial uptake of calcium is into the blastocoel (Decker and Lennarz, 1988; Nakano et al., 1963;
27 Stumpp et al., 2012; Tellis et al., 2013), although direct uptake of calcium into epithelial cells
28 has also been postulated (Mintz et al., 1981). In vitro studies show that the primary mesenchymal
29 cell bodies accumulate calcium before it is deposited in the spicule (Wilt et al., 2008). We
30 therefore raise the possibility that the filopodia that extend into the blastocoel are actively
31 sequestering calcium from the dissolved ion pool in the blastocoel and are using these ions for
32 producing the mineral granules that we observe. These mineral granules are then transported to
33 the PMCs and the epithelial cells. The transport process may be enhanced by the unusual
34 mineral-bearing multivesicular vesicles that we observed associated with the filopodial network.

Alternatively or in addition, the ions from the blastocoel may be extracted by the cells (epithelial / PMCs) and then transferred to the spicules or to other cells by the filopodia.

The cryo-FIB-SEM 3D analysis of the multivesicular vesicles proved that they do not have a nucleus, meaning that they are not cells. The multivesicular vesicles contain organelles of around 500 nm diameter, which is similar to the diameter of the dynamic calcein-labeled vesicles observed using fluorescence microscopy, and of some of the ACC containing vesicles observed intracellularly in the PMCs and in the epithelial cells (Beniash et al., 1999; Vidavsky et al., 2014). The multivesicular vesicles that we observed differ from other reported multivesicular bodies (Kornfeld and Mellman, 1989; Luzio et al., 2003) mainly in size ($>1\mu\text{m}$) and location. The multicellular vesicles we observed are inside filopodia and not only inside cellular bodies. Raman analysis shows that they contain calcium carbonate. This is consistent with their possible function in calcium transport to the spicule compartment and possibly the epithelial cells. However, we cannot rule out the possibility that the multivesicular vesicles that we observe are related to the well documented multivesicular bodies, which are abundant cellular organelles that take part in many cellular processes (Kornfeld and Mellman, 1989). These latter bodies play a role in the cellular endocytic, biosynthetic or autophagic pathways (Luzio et al., 2003).

Mineral formation is widely thought to be carried out by cells in close proximity to the tissue being mineralized: eg primary mesenchymal cells in the case of spicule formation in echinoderm embryos, and osteoblasts in the case of vertebrate bone formation. PMCs and osteoblasts are known to form membrane-bound intracellular deposits. Recently it has been shown that intracellular mineral deposits form at some distance from the forming mineralized tissue, in both sea urchin embryos (Vidavsky et al., 2014) and forming bones (Mahamid et al., 2010). It is also well known that many organisms, including organisms that do not produce mineralized tissues, form mineral deposits within diverse tissues (Brown et al., 1981). Some of these deposits are thought to function as temporary storage of ions needed for various metabolic purposes, including, where appropriate, mineralized tissue formation, whereas others are clearly involved in removing unwanted ions (detoxification). Very little however is known about the transport of mineral from one location to another within an organism. Here we show that in the developing sea urchin embryo, such transport occurs via the filopodial network located in the blastocoel.

Conclusions

Mineral-bearing vesicles inside the highly active filopodial network move rapidly in 3D for long distances, whereas mineral bearing vesicles located inside epithelial cells and PMCs move short distances. Individual vesicles were not observed to exit the epithelial cells. We also observed multivesicular vesicles associated with the filopodial network that contain mineral. The active filopodial network serves as a highway for transporting vesicles, including mineral bearing vesicles, some of which may well be the source of mineral for the spicules. The mineral in the vesicles located inside epithelial cells may also contribute to spicule formation, but based on their distinct movement pattern, may be used for different metabolic functions, other than spicule formation.

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Figure legends

Fig.1

Transmitted (A) and fluorescence (B) light images of a prism-stage sea urchin embryo continuously grown and labeled in calcein since fertilization. Images were obtained using the Deltavision system. The spicules (S) are calcein-labeled. The body cavity of the embryo is densely packed with an active filopodial network connecting cells and tissues. PMCs

(arrowheads) extend filopodia in different directions (A, marked 1-3). Calcein-labeled granules appear along the filopodial network (B, arrow).

Fig. 2

The embryonic filopodial network is packed with dynamic calcein-labeled granules of micrometer-size that are intracellular (arrows). (A) Transmitted light, and (B) fluorescence light images obtained using the Deltavision system. The spicule (S) is located on the left side of the images. Correlative fluorescence (C), Raman map (D), and Raman spectra (E) of the spicule surroundings are shown. The characteristic Raman spectra and maps (D, E) are presented using arbitrary colors: Blue – calcite, Green – proteins, Red – carotenoids. The calcein-labeled spicule (S) correlates with calcite (Fig D, E blue). A micrometer size calcein-labeled granule composed of calcite (approximately 2 μm in diameter, C, D, arrow) is most likely due to beam-induced crystallization of an amorphous calcium carbonate phase. This calcium carbonate granule is surrounded by a body of approximately 7 μm in diameter, composed, according to the Raman spectra, of proteins (D, E, green) and carotenoids (D, E, red). Carotenoids are abundant pigments in the sea urchin embryo (Kelly and Symonds, 2013). The protein characteristic Raman shifts of amides appear at 1650, 1550, 1300 cm^{-1} . The carotenoid characteristic Raman shifts appear at 1008, 1159, and 1525 cm^{-1} .

Fig. 3

High resolution imaging of the spicule environment of high pressure frozen sea urchin embryos unstained and unprocessed. (A) Cryo-SEM micrograph of a spicule (S) surrounded by cellular extensions, possibly part of the filopodial network. The spicule environment is packed with vesicles. What seems to be a filopodial extension of 1 μm diameter is marked with a rectangle and magnified in Fig. 3B. A vesicle is observed inside the fractured filopodial extension (arrow). (C) Cryo-FIB-SEM image of the spicule cross section (S) with surrounding cells. The contrast is obtained solely from differences in the InLens SE detection signal between water-rich and lipid-rich structures. Voxel size: 10nm x 10nm x 20nm, at 1.8 kV acceleration potential. Since the samples are high pressure frozen in the native state there is no signal contribution due to staining, processing or fixation. Various textures and morphologies of membrane enclosed organelles are observed; White arrowheads: very abundant, of uncertain nature; black arrowheads: membrane-

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4 enclosed sacs that when followed in 3D reach close proximity to the nucleus envelope -most
5 likely endoplasmic reticulum, black arrows: most likely mitochondria, N- nuclei. Many vesicles
6 are located outside the cell bodies (white arrows).
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10 **Fig. 4**

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13 (A) Cryo-FIB-SEM image, showing a large field of view that includes the spicule cross sections
14 (asterisks), adjacent PMC cells and their organelles, and epithelial cells (Ep). Vesicles of
15 submicron diameters dispersed in the blastocoel (arrows), and smaller features with diameters of
16 ~100nm are observed (arrowheads). Voxel size: 12.5nm x 12.5nm x 25nm, at 1.45 kV
17 acceleration potential.
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23 (B) 3D reconstruction of the region marked in (A) obtained by using manual segmentation. The
24 different features are described using arbitrary colors: filopodial extension – red, granules– pink.
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28 **Fig 5**

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30 (A), (B) Transmitted and fluorescent light images obtained using the Deltavision system.
31 Calcein-labeled granules of micron-size are packed inside a larger body, the size of a few
32 microns (arrow). (C, D) Correlative fluorescence-Raman image and spectrum measured *in vivo*
33 showing a calcein-labeled body of size 7 μm (arrow), and the Raman spectrum taken in the same
34 area, showing the characteristic peaks of calcium carbonate (calcite).
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43 **Fig 6.**

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45 (A, B) Cryo-SEM micrographs of high-pressure frozen and freeze-fractured sea urchin embryos
46 showing a spherical body that contains smaller vesicles, which we call multivesicular vesicles
47 (MVV). The MVV is inside a cellular body. N – Nucleus. (C) The 3D reconstruction of the
48 MVV in (D-H) was obtained using manual segmentation. (D-H) Cryo FIB-SEM images of a
49 high pressure frozen and freeze fractured embryo, showing slices of the MVV volume lacking a
50 nucleus. Voxel size: 12.5nm x 12.5nm x 25nm, at 1.45 kV acceleration potential. The MVV was
51 imaged in 55 slices, 25nm slice thickness: (D) slice 0/55, (E) slice 14/55, (F) slice 28/55, (G)
52 slice 41/55 and (H) slice 55/55.
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Fig. 7

Tracking of the calcein-labeled particle pathways inside the sea urchin embryos with time. (A) Transmitted light image (Deltavision) of a sea urchin embryo continuously labeled with calcein-blue. The tracking was made from a movie (see movie in SI1) taken on one acquisition plane, 20 time points, at 10s intervals. Color code for particle location: orange- epithelial cells, blue – PMCs, green – inside filopodia, red – inside filopodia in the vicinity of the spicule. (B) Transmitted light image obtained using a confocal spinning disc microscope of a sea urchin embryo labeled with calcein for 13h starting at 26h after fertilization. The tracking was made from a movie (see movie in SI4) taken on 4 acquisition planes, 81 time points, at 15s intervals. The epithelial layer outline is marked with a white line. The tracks of the different fluorescent particles are marked with different colors: epithelial cells – orange, other particles – purple and pink. The last time point of the track is labeled with the particle number. The tracking was performed using the MTrackJ program in 4D (Meijering et al., 2012). S- spicule, Ep – epithelial cells. (C-E) Example of the particle tracking in the area marked in B. Each arrow (white, black, arrowhead) indicates a specific fluorescent particle monitored with time. The detected particles are colored with green for emphasis. Same scale bar for C-E.

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Figure 1

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