Use of the Confocal Laser Scanning Microscope in Studies on the Developmental Biology of Marine Crustaceans

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KEY WORDS zooplankton; copepod; decapod; reproduction, fluorescent probes

ABSTRACT Confocal Laser Scanning Microscope techniques have been applied to study the developmental biology of marine copepods and decapod larvae. The lipophylic probes DiI and $DiOC_6$ were used to study both the external and internal morphology of these crustaceans, whereas the same $DiOC_6$ and the specific nuclear probe Hoechst 33342 were used to study embryonic development of copepods in vivo. To distinguish viable from non-viable copepod embryos, the vital dye dichlorodihydrofluorescein diacetate (H_2DCFDA) was used. Major advantages and difficulties in the use of these non-invasive techniques in studies of the reproductive biology of marine crustaceans are discussed. *Microsc. Res. Tech. 60:458–464, 2003.*

INTRODUCTION

In the last decade, conventional epifluorescence microscopy, used for immuno-cytochemical localisation of molecules and organelles in cells, has been replaced by the confocal laser scanning microscope (CLSM). The confocal principle in itself was first invented by Marvin Minsky in 1955 and later patented in 1961 (Minsky, 1961, 1988). However, the first CLSM was commercially launched only in 1983 (Boyde, 1995).

The success of the CLSM is due to some of the unique characteristics of this instrument such as the confocality, which implies that illumination, specimen, and detector have the same focus (see Diaspro, 2002; Pawley, 1995, and references therein). In fact, while in conventional wide-field fluorescent microscopy the emitted light, coming from the region above and below the focal plane, is collected by the objective lens and contributes as an out-of-focus blur to the final image, in the CLSM a diaphragm (pinhole) rejects the out-of-focus information. The final image corresponds to the point of focus into the sample. The advantage is that the fluorescence comes from a specific region of the sample and moving the focus along the depth of the sample (z-axis), several optical sections (z-series) can be acquired, without physically cutting the specimen.

In CLSM, two scan mirrors deflect a laser beam on each point of the sample exciting fluorophores into the whole specimen (scanning). Specific software can be used to reconstruct 3-dimensional (3-D) images by overlaying the in-focus z-sections (see Diaspro, 2002, and references therein). In addition to the spatial 3-D reconstructions, time-lapse series can allow a 4-D image of live specimens reconstructed over time (Diaspro, 2002) such as those required for exploring dynamic processes in living cells, as in the fertilization-induced calcium wave (Stricker, 2000).

Whereas the CLSM has found wide applications in the biological sciences and medical field (Pawley, 1995; Zhang et al., 2000), these techniques have rarely been applied in marine biological studies. To date, the only studies, to the best of our knowledge, regard the composition of marine biofilms (Norton et al., 1998), the structure of marine snow aggregates (Cowen and Holloway, 1996; Holloway and Cowen, 1997) or innervation of copepod atennules (Bundy and Paffenhöfer, 1993). Other studies have used this technique to quantify the pool sizes of plankton and detritus in aquatic ecosystems (Verity et al., 1996; Williams et al., 1995). The CLSM has also been used for studies of the reproduction and development of marine invertebrates but these have mostly regarded sea urchin embryos that have been used as a cellular model to study physiological processes during fertilisation and zygote divisions (Holy, 1999; Summers et al., 1993). To our knowledge, the only study on crustacean embryonic development using CLSM techniques was conducted by Hertzler and Clark (1992) who examined cell divisions of the shrimp embryo *Sicyonia ingentis*.

In the present study, CLSM has been employed to study embryonic and larval development of planktonic copepod species and the anatomy of decapod crustaceans. External and internal morphology are analysed using specific fluorophores that stain structures and organs, allowing for the study of their spatial distribution in whole-mount samples. In addition, patterns of mitotic divisions in copepods are followed using fluorophores to stain intracellular structures such as nuclei or yolk, and viability of copepod embryos. New experimental protocols, applied for the first time to stain marine specimens, are reported and discussed.

MATERIALS AND METHODS

Confocal Laser Scanning Microscope

Samples were observed with an inverted confocal laser scanning microscope Zeiss LSM 410 equipped with an Argon ion laser 488-nm wavelength (λ), Helium-

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Received 21 June 2002; accepted in revised form 1 October 2002
DOI 10.1002/jemt.10284
Published online in Wiley InterScience (www.interscience.wiley.com).

neon lasers (543 and 633 nm λ), and an UV laser (364 nm λ) (Coherent Inc.). Depending on the size of specimens, different magnification and objectives were used. A 40× water immersion C-Apochromat objective (Numeric Aperture NA 1.2) was used to analyse copepod embryos; 20× (NA 0.5), 10× (NA 0.30), and 2.5× (NA 0.075) plan-Neofluar objectives were used for meroplanktonic larvae. New water-immersion objectives, C-Apochromat 10× (NA 0.45) and Plan-neofluar 25× (NA 0.80), were used to study copepod larval stages. The Zeiss 410-LSM used in this study was equipped with three photomultipliers to detect three dyes simultaneously.

Each sample was optically z-sectioned with a thickness determined by the pinhole diameter, the NA of the objective lens, and the fluorescence intensity of the sample. Acquired images were 3-D reconstructed with the Zeiss and Crisel Instruments software packages MetaVue (Universal Imaging) and AutoVisualize-3D (AutoQuant).

Copepod Morphology

To examine the external morphology of copepods, *Calanus helgolandicus* (Copepoda, Calanoida), females were collected with a 250-μm plankton mesh net in the Gulf of Naples and transported within 1 hour to the laboratory. Mature females were sorted and maintained in 100 ml filtered seawater until egg deposition. Spawned eggs were isolated and allowed to hatch in a 20°C thermostatic chamber. Hatched nauplii and copepodite larvae were reared with phytoplankton cultures (Carotenuto, 1999), collected at different developmental stages, and fixed as reported in Carotenuto (1999). After a dehydration series in ethanol, larval stages were stained with 30 $\mu g \cdot ml^{-1}$ of DiI (Dioctadecyl-tetramethylindocarbocyanine perchlorate) solution (Molecular Probes, Inc.). Samples were rehydrated in a crescent graded series of bidistilled water:ethanol solution and observed with the Zeiss-410 CLSM using a 488 nm λ excitation laser. Naupliar and copepodid stages were observed with water immersion objectives 10× and 25×. Digital zoom was used at times to observe key-structures useful to identify naupliar or copepodid stages (Carotenuto 1999).

To examine the internal morphology, adult females of *Centropages typicus* (Copepoda, Calanoida) were collected in the Bay of Naples as reported above. Mature females were sorted and incubated in 25 ml SW and 25 μl of 0.5 $mg \cdot ml^{-1}$ $DiOC_6$ (Molecular Probes, Inc.) dissolved in ethanol, to obtained a final concentration of 0.5 $\mu g \cdot ml^{-1}$ (Ragnarson et al., 1992). After 30 minutes, females were sacrificed and observed with the CLSM using 488 nm λ excitation laser.

Larval Decapod Morphology

Hippolyte inermis decapod larvae, obtained from gravid females sampled in a *Posidonia oceanica* meadow off the Island of Ischia in the Gulf of Naples, were reared in 1,000-ml filtered sea water (0.25 μm) flasks (Zupo and Buttino, 2001). Larvae, at different developmental stages, and adults were fixed in 4% glutaraldheyde. After a dehydration series in ethanol, as reported in Zupo and Buttino (2001), samples were incubated in a 70% alcohol solution with 200 μL of DiI (Molecular Probes) at a final concentration of 30 $\mu g \cdot ml^{-1}$. After 72 hours, samples were washed in deionised water and observed with a Zeiss 410 CLSM using an He/Ne laser (543 nm). Images were 3-D reconstructed in a depth coloured scale with the Zeiss software.

Embryonic Development in Copepods

To follow embryonic development, mature females of the copepod *Centropages typicus* were incubated in filtered seawater until egg deposition. Soon after egg laying, embryos were collected and stained with 50 $\mu g \cdot ml^{-1}$ of the vital dye Hoechst 33342 (Sigma Chemical) for 20 minutes to stain nuclei, and in 0.5 $\mu g \cdot ml^{-1}$ $DiOC_6$ (3,3′-dihexiloxacarbocyanine iodide) (Molecular Probes) to stain cytoplasm. Embryos were observed at the CLSM at different development times with a 364-nm λ UV laser, to detect Hoechst, and with Argon 488-nm λ laser to detect $DiOC_6$ fluorescence. Bright field images, corresponding to a single in-focus plane, were acquired with a 633 nm λ He/Ne laser.

To determine the viability of copepod embryos, *Calanus helgolandicus* females were incubated in 100-ml crystallizers and maintained overnight at 20°C. After 12 hours, embryos were collected and incubated 30 minutes in 2.5-ml petri dishes containing 0.8 $U \cdot ml^{-1}$ chitinase enzyme (E.C. 3.2.1.14 from *Serratia marcescens*; Sigma Aldrich) dissolved in sea water. Embryos were then incubated in 7.5 μM 2′-7′-dichlorodihydrofluorescein diacetate H_2DCFDA (Molecular Probes) for 1 hour at room temperature in the dark. Embryos were rinsed three times in sea water and observed at the CLSM with a 488-nm λ Argon laser to detect H_2DCFDA fluorescence, and with a 543-nm λ He/Ne laser to detect autofluorescence of granules.

RESULTS

Copepod Morphology

Copepods are the most abundant organisms among the marine zooplankton; their larval development is characterized by 6 naupliar and 5 copepodid stages followed by the adult. To study the external morphology of each larval stage, the lipophilic probe DiI (Molecular Probes) was used, which penetrates tissues and organs, labelling cells in a non-selective manner. The result is that the whole organism becomes fluorescent and emits a green light when excited with an Argon 488-nm λ laser. This technique allows for the identification of key structures distinctive of each larval phase. Figure 1 shows fluorescent images of different developmental stages of the copepod *Calanus helgolandicus* stained with DiI. Figure 1A shows a ventral image of the second naupliar stage. The undivided, pear-shaped body with two long feelers in the terminal part of the abdomen, is typical of the naupliar II stage (NII). Only three appendages are developed and the mouth region appears as a protuberant structure. Figure 1B shows the NIII stage in a ventral position. Four spines in the terminal part of the abdomen are characteristic of this stage and are visible in the digital zoom (Fig. 1C). The DiI probe also stained two ventral nerve chords and two pairs of longitudinal muscles. A fluorescent image of the adult female in dorsal view is shown in Figure 1D. The genital somite, which distinguishes the mature female, and metasome segments are well delineated as are the dorsal muscles and lateral portion of the gonads.

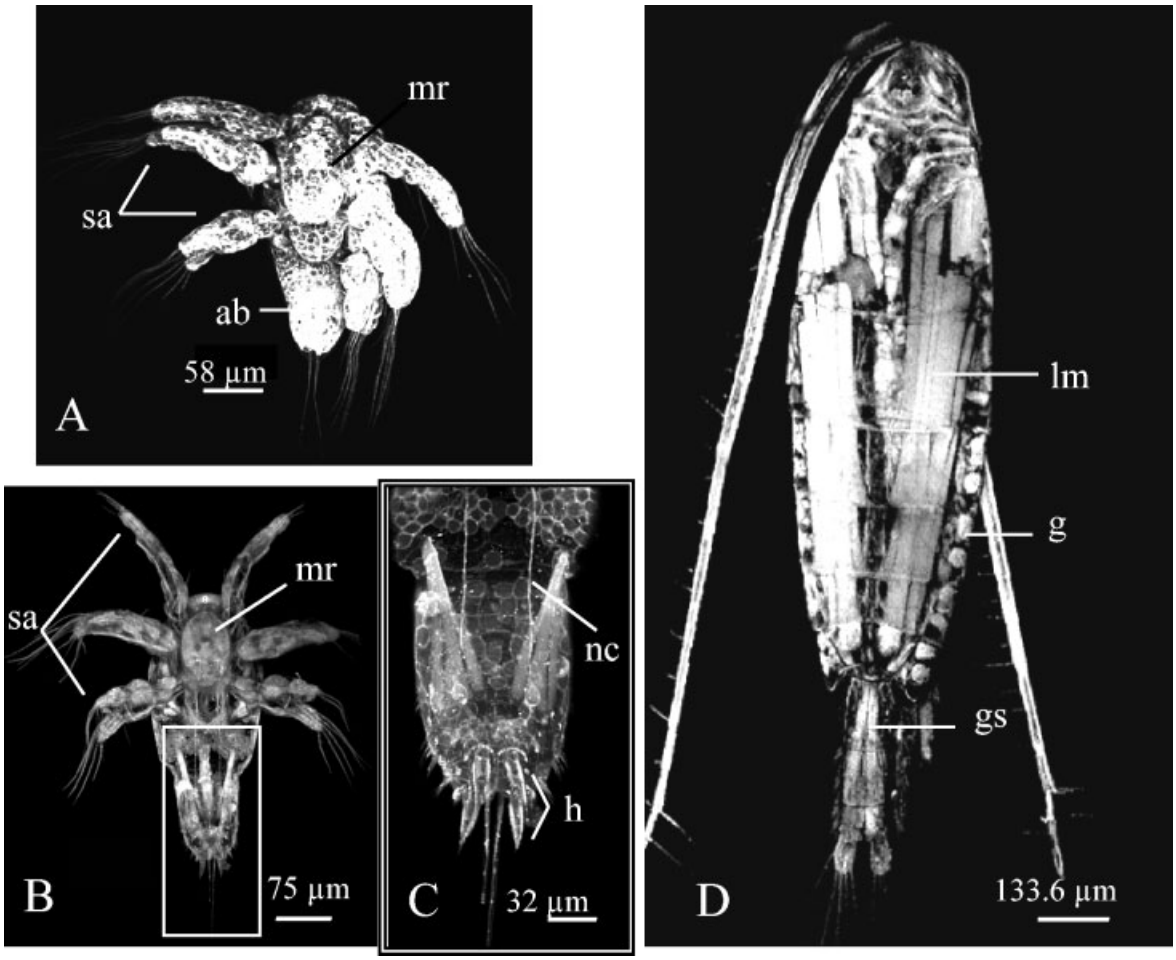

Fig. 1. *Calanus helgolandicus* larval stages stained with DiI and analysed with a Zeiss Confocal Laser Scanning Microscope, excitation with a 488-nm wavelength Argon laser. **A:** 3-D reconstruction of the naupliar NII stage, ventral view, obtained from 48 single optical sections of 2.4-µm depth. **B:** 3-D reconstruction of the naupliar NIII stage, ventral view, obtained from 27 single optical sections of 3-µm depth. **C:** 3-D reconstruction of boxed region in B obtained from 1.6 digital zoom magnification. Final image is obtained from 55 single optical sections of 1.4-µm depth. **D:** 3-D reconstruction of the adult female from 39 single optical sections of 10-µm depth. Ab = abdomen; g = gonad; gs = genital somite; h = hooks and spines on caudal rami; lm = longitudinal muscles; mr = mouth region; nc = nerve cord; sa = swimming appendages.

The membrane-permeant cyanine $DiOC_6$ was used to stain the internal organs of another copepod species *Centropages typicus* (Fig. 2). As in the case of the DiI probe, this cyanine stains lipidic structures and organs and may represent a useful probe to visualise gonadal development and oocyte maturation in copepods. Figure 2A shows a schematic representation of the internal anatomy of an adult female copepod, which shows that the dorsal region is mainly occupied by a large ovary containing oocytes in different stages of maturation (modified from Marshall and Orr, 1972). Figure 2B shows a fluorescent, 3-D reconstructed image of the thoracic region that corresponds to the cephalosome (including the first segment) and the second thoracic segment. Large, mature oocytes are visible in the dorsal diverticula of the oviduct; nuclei are not fluorescent whereas cytoplasmic yolk is well stained. The posterior genital site of the ovary, in contrast, shows a number of immature oocytes that are poorly stained due to the small amount of yolk in this previtellogenic phase (Blades-Eckelbarger and Youngbluth, 1984). Pairs of dorsal, longitudinal muscles are also stained and cover part of the oviduct.

Larval Decapod Morphology

Carbocyanine DiI was also used to follow the morphological evolution of the main organs, during the development of the decapod crustacean *Hippolyte inermis* (Natantia). Figure 3 shows a 13-day-old larva stained with DiI and 3-D reconstructed with a depth-coloured scale. The pseudocolours, from blue to red, represent the farthest or uppermost layers from the lens, respectively, and indicate the spatial relationship between organs, in a non-sectioned sample. Nervous structures in the eyestalk, digestive gland, and circulatory system, are well stained. At this larval stage, the heart and gill vessels are well developed and the circulatory system is complete. Eyestalk structures will be further modified during development until typical eye

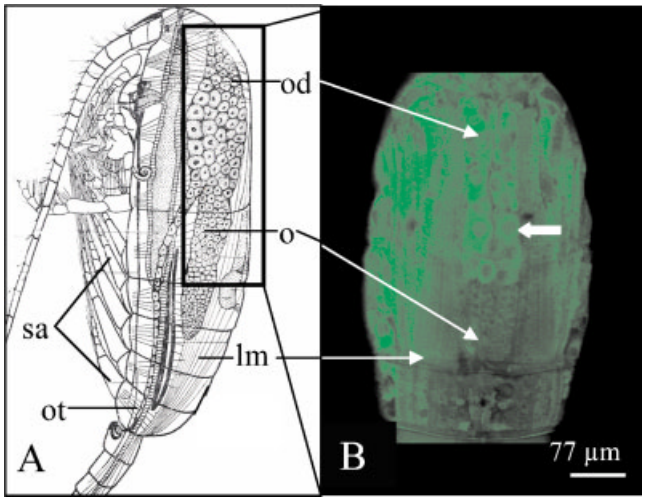

Fig. 2. **A:** Schematic representation of the internal anatomy of copepod (modified from Marshall and Orr, 1972). **B:** Dorsal view of the cephalothorax of *Centropages typicus* stained with $DiOC_6$ and analysed with a 488-mn wavelength Argon laser. The final image is a 3-D reconstruction obtained from 28 single optical sections of 5-µm depth. Thick arrow indicates mature oocytes in dorsal diverticula of the oviduct. Lm = longitudinal muscles; o = ovary with immature oocytes; od = oviductal diverticula; ot = oviduct; sa = swimming appendages; the green colour is a digital representation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

elongation is observed in the adult (Zupo and Buttino, 2001).

Embryonic Development in Copepods

The same membrane-permeant vital fluorescent dye $DiOC_6$ and Hoechst 33342 were used to stain cytoplasm and nuclei, respectively, in in vivo copepod embryos, to follow the pattern of mitotic divisions in *Centropages typicus*. Figure 4A shows vital embryos 120 minutes after egg deposition. Spines appear on the egg surface soon after spawning. Four blastomers are well visible at this embryonic stage, with nuclei (blue) at the centre of cells. Mitotic divisions are symmetrical with the morula stage appearing 240 minutes after egg deposition; blastomers are small and with a reduced quantity of yolk as indicated by the green fluorescence (Fig. 4C). In bright-field microscopy, *C. typicus* embryos are dark and opaque, and it is thus difficult to distinguish the different embryonic developmental phases (Fig. 4B and D).

Figure 5 shows a *Calanus helgolandicus* embryo positively stained with H_2DCFDA, a fluorescent probe to detect cell viability. In fact, intracellular esterases in viable cells hydrolyse this molecule that is converted from a non-fluorescent to a fluorescent form that emits green light when exited with a 488-nm λ Argon laser (Fig. 5A). On the contrary, in non-viable embryos, esterases are not activated and cells appear non-fluorescent (Fig. 5B). Autofluorescence of embryonic granules, visualized when embryos are excited with a 543-m λ He/Ne laser, is induced by incubation in chitinase enzyme.

DISCUSSION

To date, the external morphology of copepods has mostly been investigated with bright-field microscopy using camera lucida drawings (Lawson and Grice, 1969; 1973). This technique, however, is time-consuming and produces artefacts due to manual dissection of the sample. With the CLSM, in contrast, copepod morphology can be studied staining whole-mount organisms and images can be stored on digital supports to create an archive for each acquired image. Carotenuto (1999) was the first to use this technique to study larval development in the copepod *Temora stylifera*. We here show how the CLSM can be used to also examine various larval and adult stages in another copepod, *Calanus helgolandicus*. The power and beauty of this technique is that it allows for the visualisation of fine structures such as setae and setules, which are not easily visible with the conventional light microscope.

The internal organs of copepods are more difficult to stain. This is mainly due to the presence of an external chitinous wall that represents an impermeable barrier for most dyes. Also, fluorescent probes are generally not water-soluble and are unstable in water solutions (Cullander, 1999). To stain copepod gonads in in vivo organisms, dyes were dissolved in ethanol and then added to sea water containing live copepods, at non-toxic concentrations. Since copepods are herbivorous, they were stained with $DiOC_6$ via grazing.

The same vital dye and Hoechst 33342 were also used to follow embryonic development of copepods in vivo. Vital dyes have recently been used to study functional processes occurring in living cells, such as calcium dynamic signalling during fertilization (Stricker and Whitaker, 1999) or cleavage stages of sea urchin embryos in vivo (Summers et al., 1993), but cleavage of copepod embryos has never been observed until now. Due to the multi-layered fertilisation envelope, with a chitinous wall, that is formed soon after egg deposition (Blades-Eckelbarger and Marcus, 1992; Hirose et al., 1992), dyes penetrate into embryos only if incubation occurs soon after eggs are laid, before the envelope hardens. This type of in vivo staining allows the observer to follow the temporal evolution of fluorescent organelles, such as nuclei or cytoplasmic yolk platelets, during mitotic divisions of the zygote. Even if UV lasers potentially damage living cells, batches of embryos may be stored in the dark, and observed at different time intervals. The CLSM is especially useful in studies of embryogenesis in species that spawn dark and opaque eggs, difficult to observe using conventional light microscopy, resulting in confused bright images. This technique may also be applied to study embryonic abnormalities during development that leads to hatching failure such as those reported when copepods feed on diatom diets (Poulet et al., 1995a). In this study, we used a new technique to distinguish between viable and non-viable copepod embryos. Before staining with the specific probe H_2DCFDA, in vivo embryos were incubated with chitinase enzyme, to dissolve the chitinous wall. The protocol allows for the staining of copepod embryos even when the external wall becomes hardened and zygotes are in an advanced stage of mitotic divisions. Incubation in the enzyme solution does not kill embryos, and in fact nauplii are able to hatch from eggs incubated both in chitinase and H_2DCFDA probe (data not shown). This simple test to distinguish between viable and non-viable embryos can be a useful

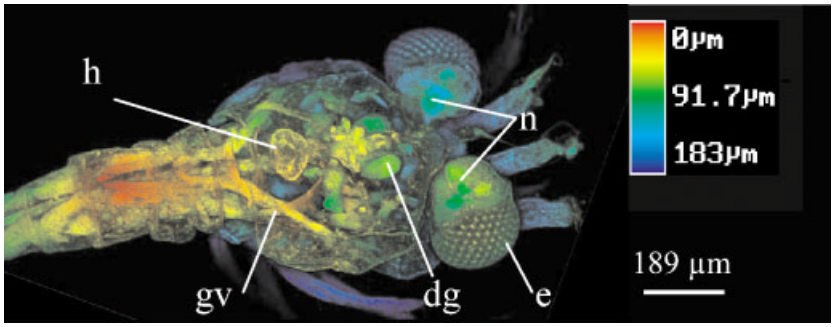

Fig. 3. 3-D reconstruction of a 13-day-old zoae of *Hyppolite inermis* stained with DiI and observed with the Zeiss confocal laser scanning microscope, 488-nm wavelength Argon laser. 3-D reconstruction, with a depth-coloured scale Zeiss software, has been obtained from 40 single optical sections of 7.5-µm depth. E = eyes; gv = gill vessels; h = heart; dg = digestive gland; n = nerve masses in the eyestalks (modified from Zupo and Buttino, 2001). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

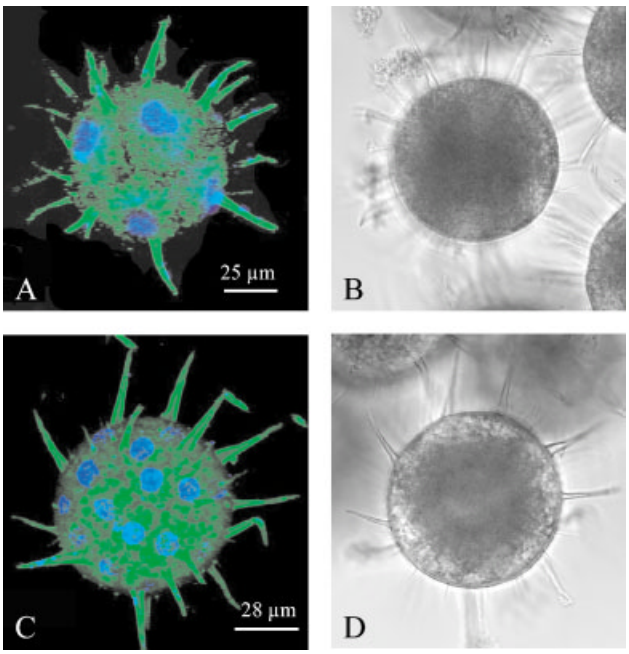

Fig. 4. *Centropages typicus* embryos stained with Hoechst 33342 (blue, nuclei) and $DiOC_6$ (green, cytoplasm) and observed with the Zeiss confocal laser scanning microscope. A,C: Fluorescent images scanned with a 364-nm wavelength UV laser to detect nuclei and with a 488-nm wavelength Argon laser to detect cytoplasm. B,D: Single-plane images observed with the Zeiss CLSM in bright-field, He/Ne 633-nm wavelength laser. **A:** 3-D reconstruction of an embryo, 120 minutes after egg laying, obtained from 35 optical single sections of 2-µm depth. **B:** Bright-field image of the embryo in A. **C:** 3-D reconstruction of an embryo, 240 minutes after egg laying, obtained from 27 single optical sections of 2-µm depth. **D:** Bright-field image of the embryo in C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

tool to evaluate secondary production in wild copepod populations (Poulet et al., 1995b).

Carbocyanine dye and CLSM techniques were used, for the first time, to follow ontogenetic evolution of the organs in the meroplanktonic decapod crustacean larvae *Hippolyte inermis* (Natantia) by Zupo and Buttino (2001). Until then, the internal anatomy of crustacean decapods was studied only by observing histological sections with the light or electron microscope (Harrison, 1992). With CLSM techniques, however, organs and tissues can be observed in their original position allowing organs to be studied in intact organisms. Passing from the planktonic to the benthic stage, the modified anatomical organisation of the adult shrimp may be followed with time (Zupo and Buttino, 2001). Also in this case, the major difficulty in applying CLSM techniques regards the permeability of the samples. Penetration of the dye is difficult, due to the external chitinous wall, especially in larger specimens. At times, it is necessary to mechanically or chemically remove the carapace before permabilisation with detergents. In fact, while staining protocols have been routinely used with several cell types, such as cells from the heart, lymphocytes, liver, and nervous tissue (Bkaily et al., 1999), in the marine sciences new protocols must be tested for each species. Lynn and co-workers (1993) used a protocol to prevent the hardening of the hatching envelope in shrimp embryos, to facilitate its removal before staining, and to allow fixation and penetration of fluorescent anti-bodies.

Another difficulty in working with marine organisms is due to their large size compared to the flattened cultured cells classically used in immuno-cytochemical studies. The most important advantage in the use of the CLSM is to visualize fluorescent organelles in thick samples, such as marine organisms and embryos. In fact, in whole-mount specimens deeper structures would be very difficult to visualize with conventional epifluorescent microscopy, which collects light emitted by all illuminated planes. Consequently, out-of focus signals are detected as well as the focal plane of interest. In contrast, with the CLSM only the in-focus plane is spotted and volume size depends on the pinhole aperture.

When imaging deep inside a sample, however, many aberrations occur, due to a mismatch in the refractive index. Refraction occurs at the interface between the objective medium and the sample, or in samples that contain mediums with different refractive indices. This implies that the actual and nominal focus positions may be different, with a consequent incorrect scaling of the image along the optical axis (Diaspro, 2002). Spherical aberrations result also in a loss of the signal passing through the pinhole with a reduction of resolution and signal intensity. It is, therefore, necessary to select the correct pinhole size in order to optimise resolution without reducing the fluorescent signal. To minimize aberrations due to the mismatch in the refractive index, water immersion objectives should be employed (Diaspro, 2002; Majlof and Forsgren, 1993).

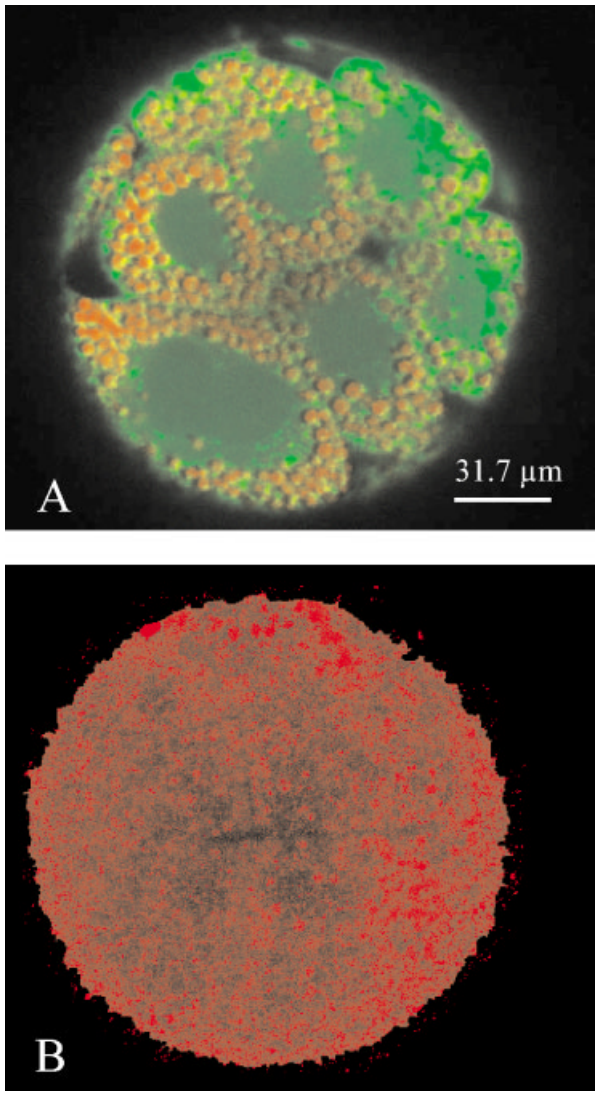

Fig. 5. *Calanus helgolandicus* embryos stained with H_2DCFDA to detect egg viability and observed with a 40× water immersion objective. Fluorescent images are scanned with a 488 Argon laser to detect H_2DCFDA (green) and with a 543-nm λ He/Ne laser to detect autofluorescence of granules (red) induced by chitinase enzyme. **A:** Single optical section of a vital embryo positively stained in green. **B:** 3-D reconstruction of a non-viable embryo, negatively stained with H_2DCFDA, obtained from 74 optical sections of 1.3-µm depth. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

Therefore, to obtain images of high quality, the objective must be accurately chosen, so as to reach a compromise between collected light and working distance in thick samples. In our studies, water-immersion objectives from 10–40× with a large NA were used, depending on the thickness of the sample. Notwithstanding these drawbacks, and the long and often pioneering work to prepare new protocols in studies on the developmental biology of marine crustaceans, the use of this non-invasive technique will surely open new frontiers in the biology of marine organisms in the near future.

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