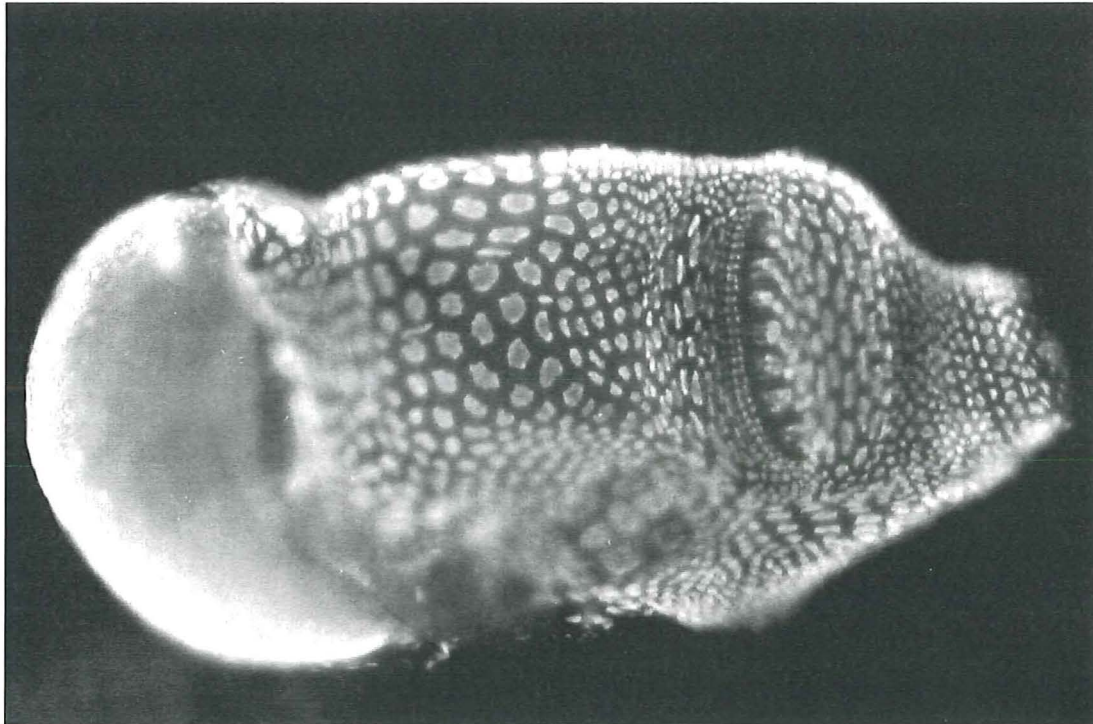


Studies on the house building epithelium of *Oikopleurid*
appendicularia (Tunicata):

Early differentiation and description of the adult pattern of
oikoplast cells.



CANDIDATA SCIENTIARUM THESIS IN CELL AND DEVELOPMENTAL BIOLOGY

Endy Spriet



Department of Zoology
University of Bergen
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Contents

Studies on the house buiding epithelium of Oikopleurid appendicularia (Tunicata):

Early differentiation and description of the adult pattern of oikoplast cells:

1.1 General introduction.....	4
1.2 References.....	8
Figures.....	9

Article A:

*Comparison of the dorsolateral oikoplastic epithelium of 5 oikopleurid
appendicularians (Tunicata): Oikopleura labradoriensis, O. vanhoeffeni,
O. dioica, O. villafrancae, O. albicans.*

2.1 Introduction.....	12
------------------------------	-----------

2.2 Materials and Methods.....	14
---------------------------------------	-----------

2.2.1 Collection of the different species.....	14
2.2.2 Fixation.....	15
2.2.3 Dissection of the epithelium.....	15
2.2.4 DNA labeling.....	15
2.2.5 Mounting.....	16
2.2.6 Microscope and photo treatment.....	16
2.2.7 Number of specimens examined.....	16

2.3 Results.....	17
-------------------------	-----------

2.3.1 Cellular patterns and cell numbers in the oikoplastic epithelium....	17
2.3.2 Bilateral symmetry of cellular patterns.....	17
2.3.3 Changes in the epithelium with the age of the animal.....	17
2.3.4 Description of the regions of the oikoplastic epithelium.....	18

The oikoplast of Fol.....	18
The oikoplass of Eisen.....	19
The field of Martini.....	19
The anterior rosette.....	19
The oblique line.....	20
The field of Leuckart.....	20
The posterior rosette.....	21
The field of Ihle.....	21
The diamond field.....	21

2.4 Discussion.....	22
----------------------------	-----------

2.5 References.....	24
----------------------------	-----------

Figures and tables.....	25
--------------------------------	-----------

Article B:

The migration of the 7 nuclei of the Eisen oikoplast of an appendicularian larvae (Oikopleura dioica)

3.1 Introduction.....	39
------------------------------	-----------

3.2 Materials and Methods.....	41
---------------------------------------	-----------

3.2.1 Animals.....	41
--------------------	----

3.2.2 Culture.....	41
--------------------	----

3.2.3 Fertilization.....	42
--------------------------	----

3.2.4 Fixation.....	42
---------------------	----

3.2.5 DNA labeling.....	42
-------------------------	----

3.2.6 Immunocytochemistry.....	43
--------------------------------	----

3.2.7 Immobilization of embryos.....	44
--------------------------------------	----

3.2.8 Microscopy and imaging.....	44
-----------------------------------	----

3.3 Results.....	45
-------------------------	-----------

3.2.1 Development of embryos.....	45
-----------------------------------	----

3.2.2 The shape and size of the polyploid nuclei of the oikoplast epithelium.....	45
-----------------------------------------------------------------------------------	----

3.2.3 Differentiation of the oikoplast of Eisen.....	46
------------------------------------------------------	----

3.2.4 Differentiation of the oikoplast of Fol.....	47
----------------------------------------------------	----

3.2.5 DNA replication.....	47
----------------------------	----

3.4 Discussion.....	48
----------------------------	-----------

3.5 References.....	49
----------------------------	-----------

Figures.....	52
---------------------	-----------

1.1 GENERAL INTRODUCTION

The appendicularians belong to the phylum Chordata, which includes the vertebrates. They form a class in the subphylum Urochordata or Tunicata. All appendicularians exhibit three features that are common to all chordates at some stage of their life: gill slits, a dorsal tubular nerve cord, and an axial rodlike notochord.

Appendicularians are small planktonic tadpole-like animals that secrete an extracellular netlike house in which they live. The houses are equipped with two types of filters, one type prevents large particles from entering the house and the other type concentrates food particles. This house is a remarkably efficient filtering device, but it also protects the animal and helps to keep it buoyant in the sea.

The appendicularians are planktonic for their entire life. They are highly transparent, and only the movement of their beating tail and the ripe opaque gonad makes them visible in the water. Appendicularians can be found in all oceans and seas, except in the Dead, Aral, Azov and Caspian Seas (Fenaux, 1966). They are most abundant in coastal waters and over the continental shelves. Generally, the appendicularians can be found in the top 100 meters of the water layer, where the phytoplankton is most abundant, the so-called euphotic zone, but a few species have been found at greater depths (400-900 metres).

There are three families of appendicularians, which are further subdivided into 14 genera and some 65 species. The three families are quite distinct in their anatomical structure (Figure 1.1). The Oikopleuridae, which are the most studied and best understood, have a short trunk and a long and narrow tail. The house encloses the entire animal and is structurally more complex than the house of the other families. The Fritillariidae have a slender and flatter trunk and a shorter and broader tail. The house is limited to a small bubble in front and underneath the animal. The Kowalevskiidae have a short trunk and a long and leaflike tail. The house resembles a deeply curved umbrella with the ellipsoid body of the animal positioned in the middle.

The house material consists of mucopolysaccharides (Körner, 1952) that are secreted on the surface of the trunk by a single layer of specialised epidermal cells, called the oikoplastic epithelium. These glandular cells are organised and fixed in definite patterns that have the appearance of a mosaic. Both this pattern and the number of cells is constant within each species. The pattern of cells within this epithelium is very complex: some regions are common to all species and other regions vary from one species to another. It is believed that the different groups of cells can secrete different layers of different density and elasticity, so that upon expansion the mucus takes a variety of shapes that contribute to the complexity of the resulting house (Figure 1.2). The diameter of these houses vary from 4 mm in diameter house of *O. dioica* to the 6-7 cm long house of *O. vanhoeffeni*.

The appendicularians are thought to feed on single celled organisms, such as bacteria, algae and protozoans. However studies have recently shown that they probably also feed on the colloidal fractions of DOC (Dissolved Organic Carbon) and POC (Particulate Organic Carbon) (Flood & Deibel, 1992).

The house contains two types of filters. The inlet filters serve to exclude particles that are too large or potentially harmful for the appendicularian. The food concentrating filter is a complex structure, made of two fluted sheets of filters kept in position by a third and intermediary layer of suspensory filaments (Flood, 1991). The mesh size of these filters is very similar between species. For example, *Oikopleura labradoriensis* has pore widths of about $0.24 \pm 0.03 \mu\text{m}$ (Flood, 1991) and *O. vanhoeffeni* has a width of $0.22 \pm 0.04 \mu\text{m}$ (Deibel & Powell, 1987). This filter is a highly efficient particle trap, and once every few seconds the animal sucks the particles off the feeding filter into its mouth through a buccal tube, and these are trapped onto the pharyngeal feeding filter.

Average-sized appendicularians can manufacture and expand one house every four hours (Fenaux, 1985). The animal will expand the new house only after it has abandoned the old one, either because the filters have become clogged with particles or because it has been attacked by a predator such as a fish larva. Once the animal has discarded the old house, it will almost immediately begin to expand a new one (Figure 1.3). The animal will start to swim in a circle, slightly expanding the new house. Then the animal will undertake a

series of violent movements to move water underneath the house rudiment (the prehouse). Whole tail contractions on alternating sides make its trunk nod back and forth, forcing water beneath the rudiment on the backstroke of the trunk. When the rudiment is sufficiently expanded, the animal pulls its tail into the house. The tail then begins with slow sinusoidal motions which pump the water into the house until it reaches full size, and the animal starts to feed.

Some appendicularians (subgenus *Vexillaria*) have bioluminescent inclusion bodies on the surface of their houses. These inclusion bodies contain grains, called lumisomes and light is emitted by mechanical stimulation of the lumisomes. In each species these inclusion bodies are located in a species-specific pattern. *O. labradoriensis* and *O. dioica* have a well organised pattern, while in *O. vanhoeffeni* the inclusion bodies are scattered all over the house (own observations).

Since appendicularians are among the few multicellular organisms that are capable of feeding on DOC, POC and nanoplankton, they hold an unusual and important position in the food web as a shortcircuiter to the microbial loop. Appendicularian predators include fish-larvae, jellyfishes, chaetognaths and siphonophores.

Appendicularians as a model organism for cell and molecular biology

Based on quantitative fluorescence measurement of DAPI and Hoechst 33258, stained gamete nuclei from *O. dioica*, compared to nuclei of known genomic sizes from other taxa, the genome size of *O. dioica* has been estimated to about 25 Mbp (Flood & Spitzer, unpublished data). This is less than twice that of *Saccharomyces*, the first eukaryote genome to be fully sequenced so far.

The small genome size, the rapid generation time of about a week at 18 degrees (own observations), the cell constancy and the accessibility of these animals, favours this organism as a possible model for cell and molecular biology.

Recent sequence analyses of about 400 nucleotides from the 5' end of 28S rRNA from two oikopleurid appendicularians revealed that «Appendicularians and vertebrates form a very robust monophyletic unit, the two oikopleura species forming a sister group to all

vertebrates» (Christen & Braconnot, in press). Therefore the appendicularia represent an interesting taxon at the transition between invertebrates and vertebrates, of relevance to many biological problems e.g. molecular evolution.

The aim of this thesis is to explore the feasibility of detailed mapping of the adult cellular patterns and to track cellular differentiation of the oikoplastic epithelium.

In «Comparison of the dorsolateral oikoplastic epithelium in five oikopleurid appendicularians (Tunicata): *Oikopleura labradoriensis*, *O. vanhoeffeni*, *O. dioica*, *O. villafrancae*, *O. albicans*.» the aim was to investigate the possibility of reliable photographic documentation and try to define homologous regions of the oikoplastic epithelium in different species. The five species used were chosen because they were available either in the fjords of Norway, or from abroad.

In «The migration of the seven nuclei of the Eisen oikoplast of the larvae of *Oikopleura dioica*», the aim was to study the differentiation of the Eisen oikoplast during ontogeny. *O. dioica* was chosen because it is the most studied species and can easily be cultured.

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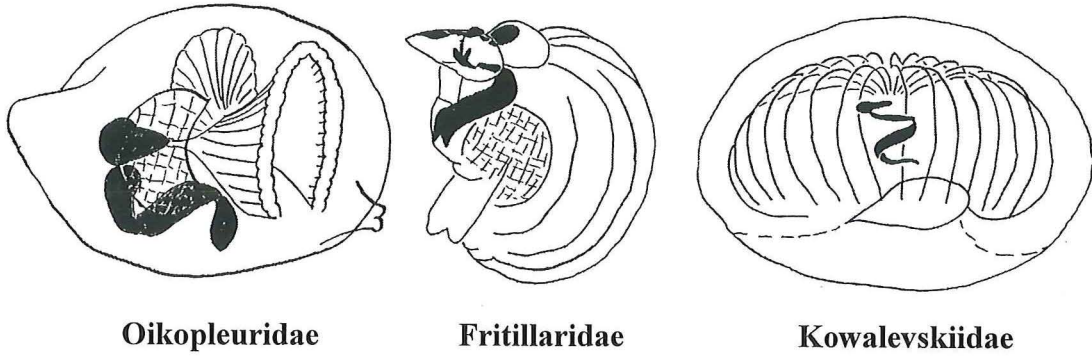
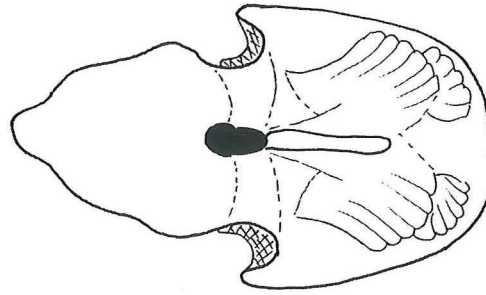
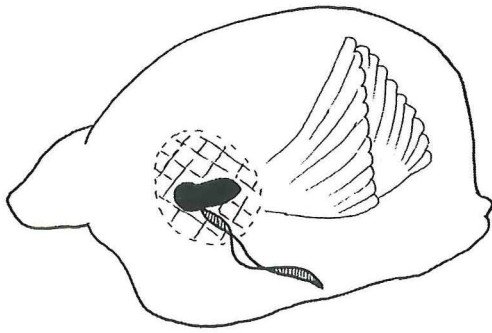
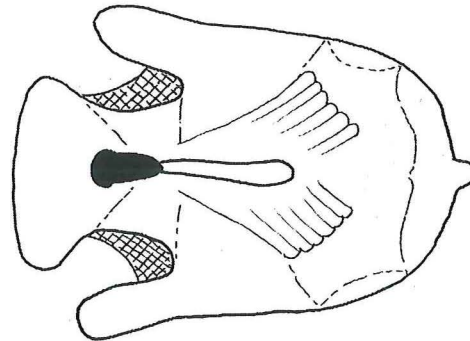
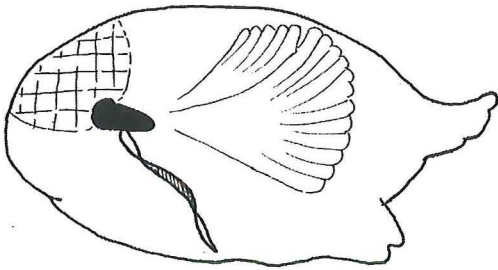


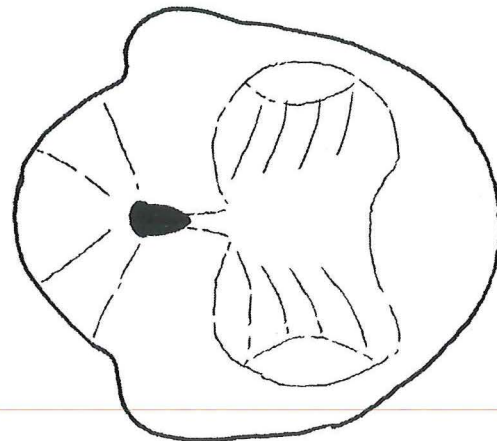
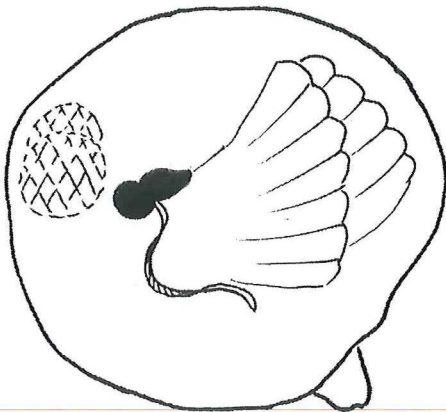
Figure 1.1
Schematic drawing of the three families of appendicularians. Animal is in black, front of the animal to the right. Modified from: Flood, 1994.



Oikopleura labradoriensis



Oikopleura vanhoeffeni



Oikopleura dioica

Figure 1.2
Schematic drawing of the house in three species from own observations. The houses on the right are seen from above, the houses on the left are seen from the side. The animal is in black. Front of the animal to the right.

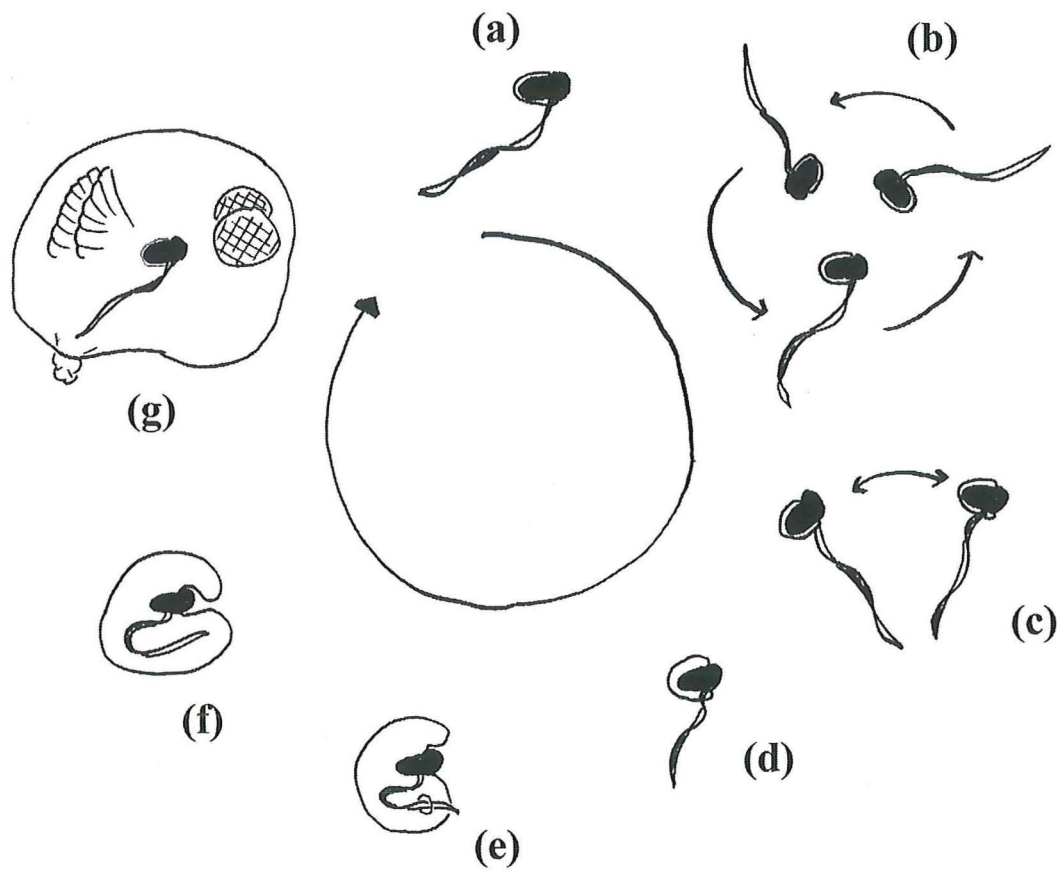


Figure 1.3

Schematic drawing of the expansion of the oikopleurid house from own observations. Once the animal has discarded the old house (a) it will soon start to swim in a circle (b), to be followed by a series of violent movements back and forth, forcing water beneath the rudiment (c). When the rudiment is sufficiently expanded, the animal pulls its tail into the house (e and f) and commences with slow sinusoidal motions, and in this way expands the new house to its full size (g).

Comparison of the dorsolateral oikoplastic epithelium in five oikopleurid appendicularians (Tunicata): *Oikopleura labradoriensis*, *O. vanhoeffeni*, *O. dioica*, *O. villafrancae*, *O. albicans*.

2.1 INTRODUCTION

The five oikopleurid species studied were originally described by Leuchart in 1854 (*O. albicans*), Fol in 1872 (*O. dioica*), Lohmann in 1892 (*O. labradoriensis*), in 1896 (*O. vanhoeffeni*), and Fenaux in 1992 (*O. villafrancae*). Unto now the appendicularians have been categorised into different species from the appearance and size of their tail, trunk, digestive tract, gonad and inclusion bodies. The five species studied in this paper are classified as Vexillarians, as they all contain buccal glands and subchordal cells (Fenaux, 1993). Descriptions of the cell pattern within their oikoplastic epithelium have so far been provided only in rough sketches, except for *O. albicans* (Fenaux, 1971).

The five species vary in size and shape, and their secreted and expanded houses are very different from each other. Each species is unique in the way the cells of the oikoplastic epithelium characterised by polyploid nuclei of remarkable shapes are arranged in different and sometimes well defined regions. So far little is understood of the secretion of their highly complex filter houses. Two regions, with the largest polyploid cells, have always been easy to recognise. These are the oikoplasts of Eisen, which are responsible for the production of the anlage for the inlet filters; and the oikoplasts of Fol, which are responsible for the production of the anlage for the food concentrating filters (Figure 2.1).

Lohmann and Bückmann (Lohmann, 1898, Bückmann, 1924, Lohmann & Bückmann, 1926) defined regions of the oikoplastic epithelium of many different species. The cellular patterns were hand drawn. Unfortunately, most of these drawings are not accurate

enough to allow a direct comparison with photographic prints of the cellular pattern, as obtained on microdissected and stained samples.

Such photographic documentation of the cellular pattern of an oikoplastic epithelium has only been shown previously for *O. albicans* by Fenaux (1971) who also subdivided the oikoplastic epithelium in a large number of regions for descriptive purpose. However, the borders between these regions are not always exactly defined.

It would be desirable to define the regions of the oikoplastic epithelium according to their distinct contribution to the secretion of the house rudiments and to the function of distinct parts of the expanded filter house. However until our knowledge about these aspects is greatly improved, a definition of distinct regions must be based on the tendency of the human eye and brain to draw borders along straight or smoothly curved lines that may appear in the cellular pattern between groups of cells with more or less distinctive appearance. Also, as long as our material is limited to material stained to reveal nuclear DNA, rather than to reveal cell borders, these borderlines are likely to relate to arrangements of nuclei rather than cells. Only in a few exceptional cases do we know the exact function of oikoplastic regions in relation to their secretion and formation of the highly complex house, these being the inlet filters, produced by the oikoplasts of Eisen and the food concentrating filters, produced by the oikoplasts of Fol.

Some cells of the oikoplastic epithelium may be actively involved in secretion for only part of the house building cycle, while other cells may secrete permanently. These cell-secreting "fates" are unknown because the "secretion" could not yet be observed. This work suggests that there are defined regions, but these regions will have to be modified when their secretory patterns and their participation in building specific regions of the complex house is better understood.

The description of the pattern is based on the work of Lohmann and Bückmann (1926), and that of Fenaux (1971). Some of the regions defined by the authors above have been modified and some regions have been added and others have been removed.

We have established a reliable photographic documentation of well defined regions of the oikoplastic epithelium in five species, to understand which parts are conserved and which are not. These observations have improved our understanding of the taxonomic relation between the species and of the rules for constituting an orderly pattern of house secreting cells in the oikoplastic epithelium.

2.2 MATERIALS AND METHODS

2.2.1 Collection of the different species

O. labradoriensis were collected at Friday Harbour (USA) and from the fjords of Hordaland (Norway). *O. vanhoeffeni* were collected and sent to us from Newfoundland (Canada). *O. albicans*, *O. villafrancae* and *O. dioica* were collected from Villefranche-sur Mer (France). *O. dioica* were also collected from Hekkingen near Tromsø (Norway). The species vary in size from 0,5mm to 3,5 mm (adults) (Table 2.1). Mostly nearly mature animals were used as they contain the most developed epithelia.

Table 2.1 Trunk length of adult animals of the different species.

SPECIES	TRUNK LENGTH IN MM	NUMBER OF SPECIMENS SUCCESSFULLY MOUNTED AND EXAMINED
<i>O. labradoriensis</i>		
from USA	1,0-1,5	} 25
from Norway	0,6-1,4	
<i>O. albicans</i>	2,4-3,5	5
<i>O. vanhoeffeni</i>	2,5-3,5	4
<i>O. dioica</i>	0,5-0,9	25
<i>O. villafrancae</i>	3,0-4,1	3

2.2.2 Fixation

The animals were commonly fixed in 2-3% formaldehyde (diluted in seawater and PBS). *O. vanhoeffeni* were fixed in 2,5% glutaraldehyde (diluted in seawater).

2.2.3 Dissection of the epithelium

The five species used in this purpose have different sizes and trunk shapes. Therefore some are easier to dissect than others, but the procedure is the same for all of them.

Firstly the size of the animals was measured (Shiga, 1976), using a Nikon stereomicroscope. The house rudiment(s) (up to seven rudiments have been reported on the trunk of one animal; Fenaux, 1985) were removed from the surface of the epithelium. This was done by using tweezers or very thin needles, sometimes with their tips deformed as hooks. It was easiest accomplished by getting hold onto the rudiment from behind, just in front of the gonad. Holding on to the tail of the animal was often useful, but it was easily torn off. After cutting away the rudiment, the gonad was removed and the rest of the internal organs (stomach, intestine, oesophagus and caecum) were then easily pulled out. The endostyle usually remained behind within the trunk, but with a steady hand this could also be removed. The oral glands usually remained in position and these were only occasionally removed.

The epithelium was in this way reduced to a barrel-like structure. With razorblades fractured as pointed knife blades, the epithelium could then be cut open ventrally from behind, all the way to the mouth. Often (depending on the species) an extra cut was needed along the sagittal plane into the anterior-dorsal region. The epithelium was then ready to be unfolded and dyed.

2.2.4 DNA labeling

Hoechst 33258 (Sigma, B2883) was used for labeling of DNA. Hoechst, which is an Adenosine-Thymine specific dye, will only intercalate with three repeated A-T pairs (Müller & Gautier, 1975).

The dissected epithelia were placed into a minimum volume of Hoechst 33258 diluted in the fixation solution. Usually a concentration of 5 µg/ml Hoechst (stock of 0,1 mg/ml in water) diluted in 2-3% formaldehyde (in seawater) was used. The epithelia were stained for 5-10 minutes. The nuclei will stain bright blue when excited with UV light at 365 nm.

2.2.5 Mounting

The oikoplastic epithelium was removed from the staining solution and mounted in Citifluor® (Ted Pella, Inc.) designed to reduce photobleaching of fluorochromes. The epithelium was unfolded by the use of thin needles. A coverslip was then applied and the surplus of mounting medium removed with blotting paper.

2.2.6 Microscope and photo treatment

The epithelia were photographed using an Olympus Vanox microscope, model AHBT3 with fluorescence attachment AH3-RFC. They were digitised and entered into a computer using a Mirascan (version 1.2) and treated in Adobe™ Photoshop (version 3.0.5).

It should be taken into account that some information was lost during the process. The original epithelia were photographed with a 10x objective (x3,13) and for the larger species, mosaic were made by putting together 2-13 separate photos. These patch-works photos were digitised in a computer and reduced in size. The brightness and contrast were adjusted and artefacts along the junctions between the photos were removed by editing the digitised images. Due to these adjustments, some minor features may have disappeared. The most apparent loss is the otherwise weak and narrow bridges between two or more segments of the same nuclei. The contralateral side of the epithelium should therefore always be taken into account. The final photographs were printed on a Canon CLC 800 LaserWriter.

2.2.7 Number of specimens examined

The number of specimens examined varied between species, as some are more easily available than others. See table 2.1.

2.3 RESULTS

2.3.1 Cellular patterns and cell numbers in the oikoplastic epithelium

The cellular patterns were defined using the ability of the eye and brain to draw borders along nuclei of similar appearance and shape. These regions of cells and the patterns of their cells, as well as the number of cells in each region and in the entire oikoplastic epithelium (including the ventral oikoplast) were constant within the species, but varied between the different species (Table 2.2).

2.3.2 Bilateral symmetry of cellular patterns

A striking bilateral symmetry was evident in the oikoplastic epithelium in all the five species examined. Not only were the nuclei number and pattern symmetrical on the two sides, but also the individual nuclei of corresponding cells showed striking mirrorlike similarities in sizes and shapes (Figure 2.2). This mirrorlike symmetry of the nuclei were however far from perfect in all regions.

2.3.3 Changes in the epithelium with the age of the animal

The characteristic shape of polyploid nuclei of the epithelium changes with the age and/or size of the animal. In all species the nuclei become larger, more complex and ramified as the animal gets larger and/or older. For example the nuclei of the oikoplastic epithelial cells in a young *O. dioica* are mostly round, but as the animal matures they become larger and more flattened. For *O. vanhoeffeni* the nuclei become larger and increasingly ramified, and right before spawning the nuclei are so ramified that it is difficult to tell two nuclei apart.

Figure 2.3 show four stages in the development of nuclei from the two right Leuckart cells of *O. labradoriensis*. In a juvenile the two nuclei are slightly elongated and clearly separated from each other and from the nuclei of surrounding cells. In a mature *O. labradoriensis* the same two nuclei have become ramified and they interdigitate.

2.3.4 Description of the regions of the oikoplastic epithelium

Figure 2.5 gives an outline of the defined regions of an unfolded oikoplastic epithelium and their names. The regions defined in this drawing are found in all five species studied. Some species have regions that are more developed than other species. *O. albicans*, *O. villafrancae* and *O. vanhoeffeni* have additional regions to those marked in figure 2.5, because some regions have evolved and produced regions of cells where nuclei have similarities to each other and are distinct from those of the neighbouring cells, thus eye-catching as a separate region.

Table 2.2 gives an overview of the regions defined in each of the five species. Following is a more detailed description of some of them.

The oikoplast of Fol

The oikoplast of Fol is divided into 4 regions: the Fol anterior region (fa), the Giant cells (gc), the Nasse cells (nc) and the Fol posterior region (fp) (Figure 2.4).

The Fol anterior region contains 35-70 cells. Their nuclei have an elongated shape and are oriented in a curved dorsoventral axis. The giant cells are localised posterior to these. These cells have more ramified nuclei that penetrate deeper into the epithelium. Their long axis are oriented anterior-posterior and most species have 8 giant cells, except *O. dioica* which has 7 (Figure 2.5c).

The Nasse cells consists of three rows of small cells lined after each other in a dorsoventral line. The nuclei of the Nasse cells have spherical (*O. dioica*) or cubical shapes. Generally, the most anterior row contains the largest cells and nuclei, while the two posterior rows may be more «squeezed» together. Behind the Nasse cells follows 10-11 rows of cells in an antero-posterior direction. Their nuclei are elongated, of different sizes and oriented along a dorsoventral curved axis. An antero-posterior line dividing the Fols oikoplast anteroposterially in two halves also result in approximate symmetry of cellular pattern between the two halves. The six anterior rows contain the same totality of cells with their nuclei arranged in exactly the same manner in all the five species. In the most anterior row there are six cells, in the second row seven, in the third row seven... (6-7-7-6-5-7).

The oikoplast of Eisen

The oikoplast of Eisen is composed of six or seven cells with large nuclei, depending on the species, and a row of small cells anterior to these called the «chain of pearls» (Figure 2.6). The nuclei of the large nuclei look similar to the giant cells of the oikoplast of Fol, though their shape is simpler. *O. labradoriensis*, *O. vanhoeffeni* and *O. albicans* have six large ramified nuclei. These are disposed in three rows dorso-ventral with two cells each in the antero-posterior axis. *O. dioica* and *O. villafrancae* have seven nuclei, the shape which is simpler than in the other three species. They are disposed in the same three rows as above, but the middle row contains three cells instead of two.

Anterior to the large cells we find a row of small round nuclei lined up like a «chain of pearls» in a dorsoventral line. The number of these nuclei are species-specific.

The field of Martini

On each side the field of Martini is recognised posterior to the oikoplast of Fol (Figure 2.4). Each of them contains seven large cells (except for *O. dioica*) located in the centre of the structure and surrounded by a few rows of elongated cells. This arrangement is similar for *O. labradoriensis* (Figure 2.10) and *O. villafrancae* (Figure 2.13). The seven larger cells can be easily distinguished as their nuclei are about twice larger than those of the surrounding cells. This is not the case of *O. vanhoeffeni* (Figure 2.11) and *O. dioica* (Figure 2.12). In these species the cells and nuclei are more or less of the same size. However all the species have 38 cells in each of the fields of Martini.

In *O. albicans* (Figure 2.14) the seven cells with larger nuclei can be easily distinguished but these are to their posterior surrounded by two-three rows of cells with nuclei of nearly the same size, though rounder in shape.

The anterior rosette

This zone forms an oval- or triangular-like area posterior to the two oikoplasts of Fol and between the two fields of Martini (Figure 2.4). It contains cells of different sizes. The centre of the rosette consist of small cells clustered together in an antero-posterior, elongated, oval structure. These cells are surrounded by 16 larger cells. This centre is essentially similar in the five species, but there are small differences in number of cells.

The anterior rosette of *O. labradoriensis* (Figure 2.7a) and *O. villafrancae* (Figure 2.7d) are arranged in the exact same pattern. The centre of the anterior rosette contains 33 cells, i.e. 17 small cells surrounded by 16 larger cells. To the lateral and anterior of the centre, 88 other cells can be counted. The total sum of the anterior rosette is therefore 123 cells.

In *O. albicans* and *O. vanhoeffeni* the centre of the anterior rosette has the same number of cells as in the two species mentioned above and is arranged in the same manner, containing 17 smaller cells surrounded by 16 larger cells. However the number and pattern of the cells surrounding the centre rosette are different. *O. albicans* (Figure 2.7e) has 62 cells (or more) around the centre, and *O. vanhoeffeni* (Figure 2.7b) has 70 cells to the anterior and lateral of the centre.

Defining the anterior rosette in *O. dioica* has been a problem, because the epithelium is very small and because the anterior rosette seems to contain a number of larger cells compared to the other four species above. Altogether the anterior rosette in *O. dioica* (Figure 2.7c) contains 110 cells, whose size is rather constant.

The oblique line

The line contains 9 cells aligned between the Eisen oikoplast and the field of Martini (or the field of Ihles in *O. vanhoeffeni*).

It contains one row of 9 large flattened cells, with their larger diameter oriented along the antero-posterior axis, while the cells are stacked on each other oriented antero-ventral to postero-dorsal, as can be seen in *O. vanhoeffeni* (figure 2.11). The cells increase in size going ventrally to dorsally. The oblique line can only be found in *O. villafrancae*, where the nuclei have simple shapes, and in *O. vanhoeffeni*, where the nuclei are highly ramified and become more complex the more dorsally situated.

The field of Leuckart

The field of Leuckart contains 1-4 conspicuous nuclei on both sides of the anterior rosette or inside the fields of the large dorsal cells (Figure 2.4).

O. labradoriensis (Figure 2.8a) has two cells on each side. One nucleus has a feather shape and the other one has an L-shape. In mature animals the nuclei are interdigitated.

O. villafrancae (Figure 2.8d) has four cells on each side. Three have elongated nuclei and one has a «thick L-like» nucleus.

O. vanhoeffeni (Figure 2.8b) has one elongated, snake-shaped nucleus on each side.

O. albicans (Figure 2.8e) has two nuclei on each side, one large and elongated nucleus (S-shaped in mature animals) and lateral to this a bended nucleus.

O. dioica (Figure 2.8c) has two elongated cells on each side.

In *O. labradoriensis*, *O. vanhoeffeni* and *O. albicans* the cells of the Leuckart region on each side of the mirror image are separated by five dorsal cells with large nuclei.

The posterior rosette

This cross-shaped structure is localised at the middorsal line near the very posterior end of the epithelium (Figure 2.9). The dorso-ventral branches are thicker and contain a larger number of small cells, whereas the antero-posterior branches contain elongated cells at each arm. The centre of the cross contains small cells. The nuclei at the posterior arm are oriented along a sagittal plane, whereas the anterior nuclei are oriented in the transverse plane. The posterior rosette of all the species (except *O. dioica*) contains 65 cells organised in a similar pattern.

The field of Ihle

The field of Ihle is a «plum shaped» field found dorsal in the oikoplast of Eisen in the epithelium of *O. vanhoeffeni* and *O. labradoriensis*. It consists of a total of 25 cells, with 2 characteristic cells having elongated nuclei.

The diamond field

This «diamond shaped» region contains 22 cells with long and thin nuclei oriented along the longitudinal axis. The diamond field is situated lateral, between the oikoplast of Eisen and the oikoplast of Fol, and is very conspicuous in *O. albicans* (Figure 2.14), but has not been identified in any other species.

2.4 DISCUSSION

The oikoplastic epithelium consists of a single layer of cells. These cells, as well as their nuclei vary in size and shape, and in general are gathered in groups with comparable geometry. Several such groups have been visually identified and given specific names in previous literature. However this tendency of the human eye and brain to group objects of comparable geometry and to draw borderlines between such groups, is rather a subjective process. The exact location of such borders in an epithelium counting approximately 2000 cells may be questioned. In the case of Oikopleurid appendicularia a number of cell groups, oikoplasts, fields etc., have been described and given specific names in previous literature. However, since these descriptions are usually based on one species, vaguely formulated and documented by handmade sketches, rather than micrographs, it is often impossible to know exactly where the borders are supposed to be drawn. As a consequence, the number of cells contained in a group may vary.

Defining the precise pattern was easily done for the oikoplasts of Fol and Eisen. For other regions, some of the cells near the borderlines were very similar and the borders therefore hard to outline. This was the case for the anterior rosette and the fields of Martini. These regions are only partly surrounded by other regions and the sum of cells has helped defining the exact regions.

There is a striking epithelial pattern homology between the species. *O. dioica* and *O. villafrancae* have similar oikoplasts of Fol and Eisen in the shapes of their nuclei and in the number of cells. *O. labradoriensis*, and *O. villafrancae* have identical anterior rosettes. They, as well as *O. albicans*, have also identical number and arrangement of nuclei in the fields of Martini. The posterior rosette is more or less identical in all the species examined, except for *O. dioica* which have a poorly defined posterior rosette. The fields of Leuckart, with their conspicuous cells, show homology in only *O. labradoriensis*, *O. vanhoeffeni* and *O. albicans*. Though the first and latter contain two cells in each field, while *O. vanhoeffeni* only has one, these cells are situated in exactly the same position relative to the dorsal medial plane.

Studying an appendicularian house has been difficult and speculative. Their structure is poorly understood because their houses are too fragile to be collected by nets or handled and preserved in the laboratory without damage (there are exceptions). The houses are transparent and it's no use trying to preserve the house for later morphological studies, as it deflates when the animal is separated from its house. The house can therefore only be studied while the animal is inside and beating it's tail. Most species can not be held in small containers and photographed, because when the animals sense an obstacle or obstructed water circulation, they simply escape from their houses. Therefore it has not been possible to approach the secretory function of each of the regions of the epithelium. I believe that the function of some regions of the oikoplastic epithelium can be identified by comparing homologue structures of the houses from the different species compared with the regions of their epithelium.

By comparing the sketches of the two houses of *O. labradorensis* and *O. vanhoeffeni* in figure 1.2, one can see that the «lateral cushion chambers (LCC)» (Flood, 1990) in *O. vanhoeffeni* are much larger than in *O. labradorensis*. The lateral cushion chambers of the house probably have their origin in the fields of Ihle of the oikoplastic epithelium. The field of Ihle is situated posterior to the oikoplast of Eisen, which is responsible for the inlet filter situated near the LCC in the house. The lateral cushion chamber is more developed in *O. vanhoeffeni* and could therefore account for the structural differences in the house.

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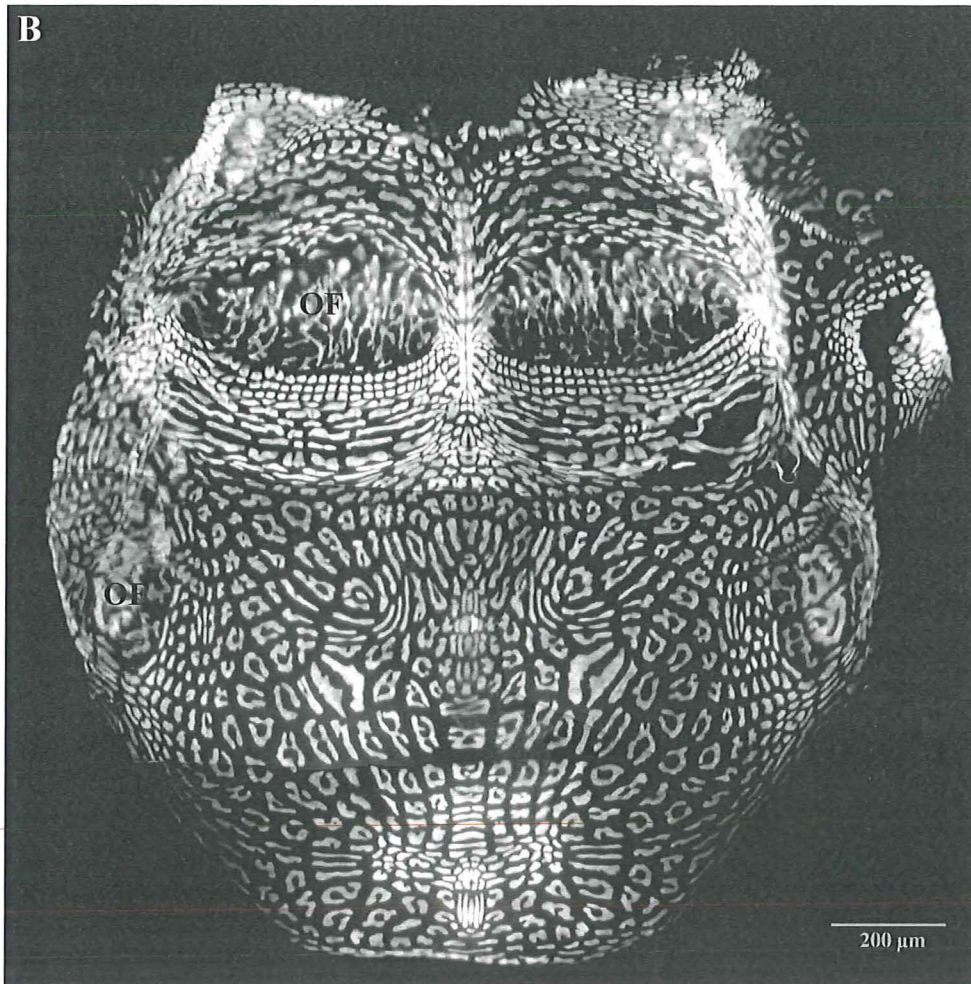
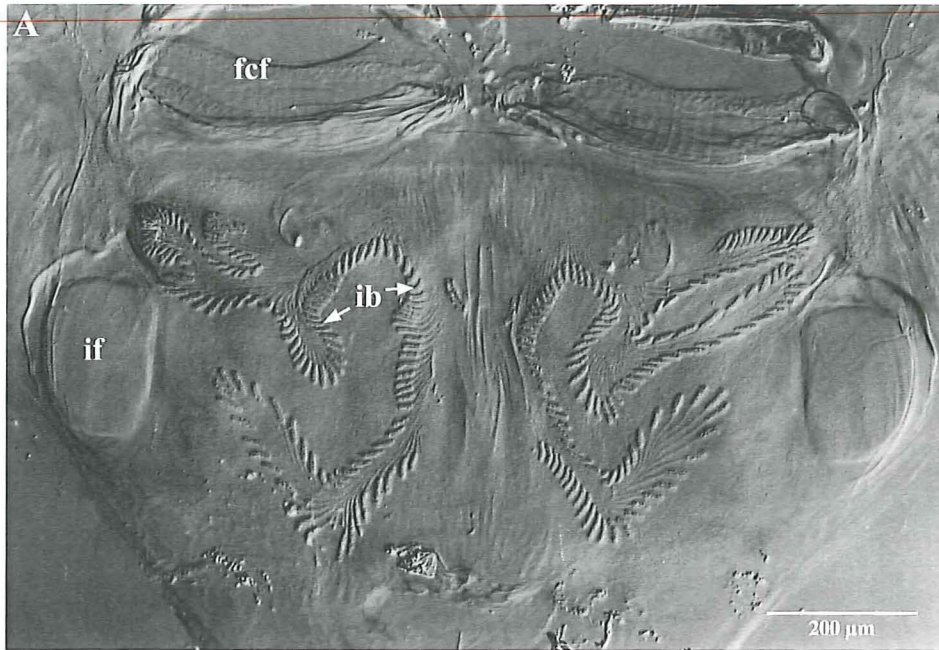


Figure 2.1
 A) House rudiment and inclusion bodies (ib) of *O. labradoriensis*. The food concentrating filter (fcf) and the inlet filter (if) are easily recognized. B) The oikoplastic epithelium of *O. labradoriensis*, unfolded and labeled with Hoechst. OE: Oikoplast of Eisen, OF: Oikoplast of Fol.
 Scale: 200 μm.



Figure 2.2
The bilateral symmetry is evident in the oikoplastic epithelium.
This is taken from *O. labradoriensis*.

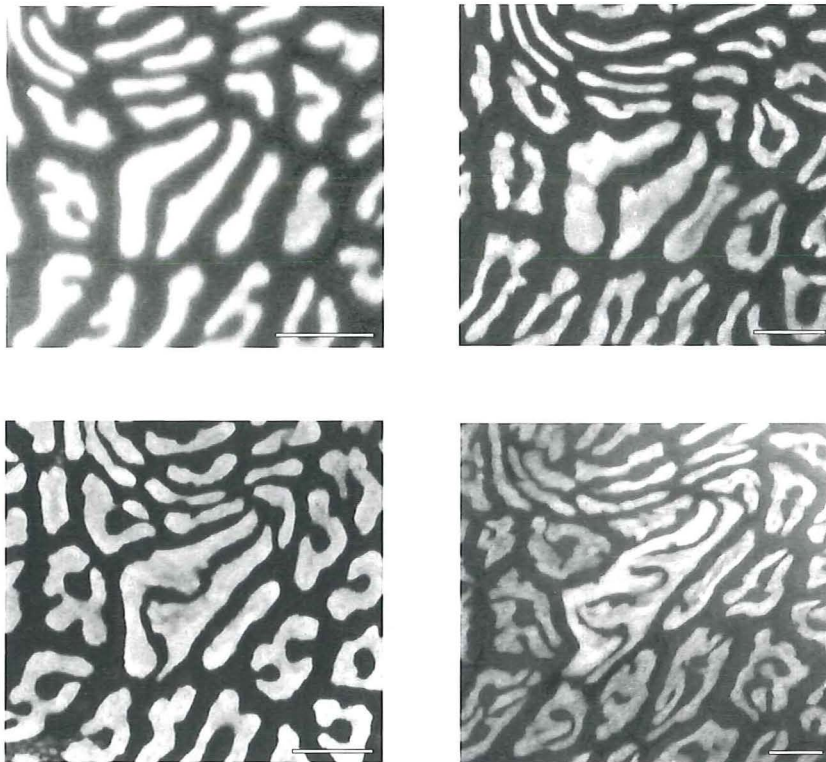


Figure 2.3
The cells of Leuckart from *O. labradoriensis* at four different stages of maturation.
Scale: 50 μm .

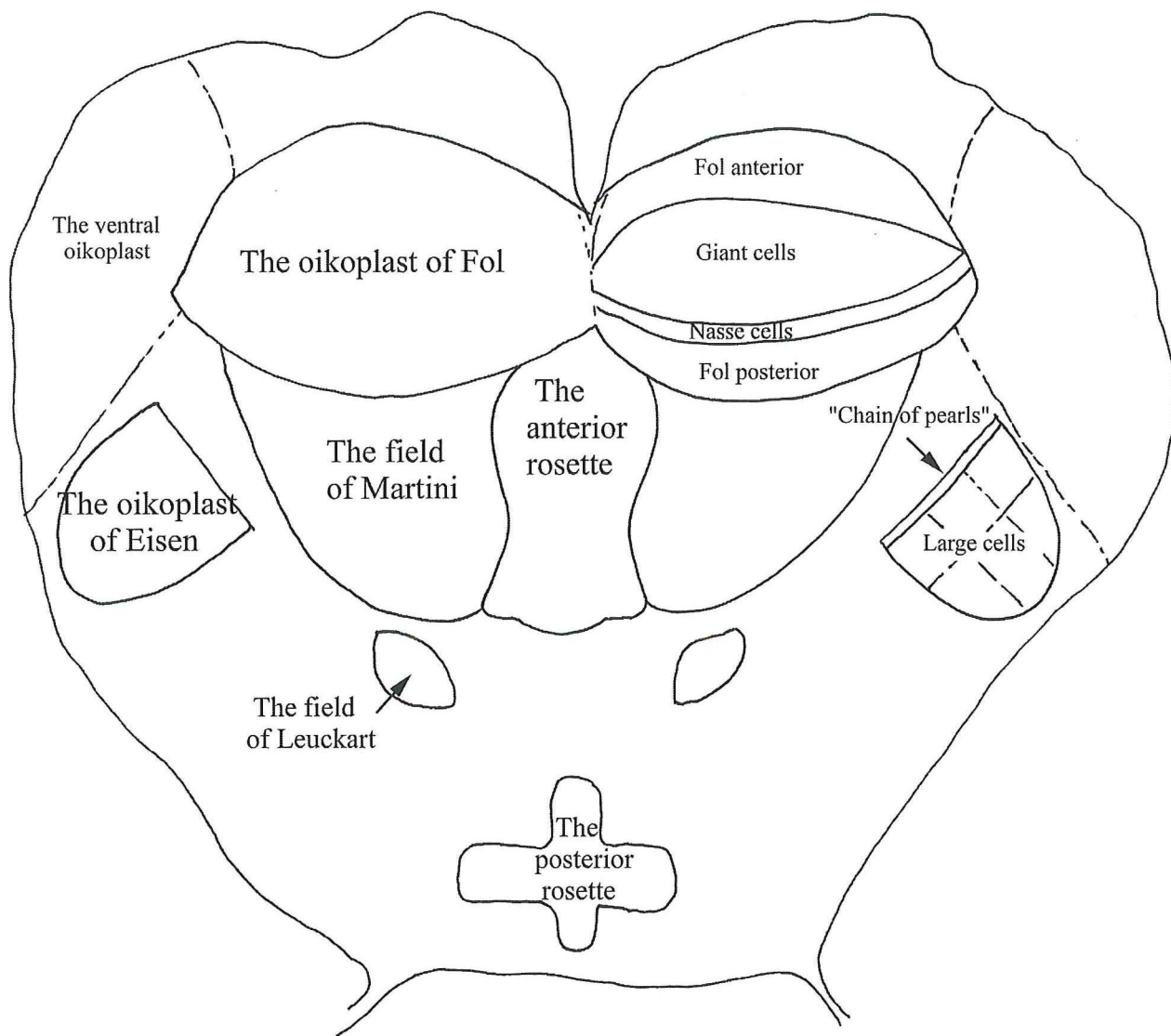


Figure 2.4
 Diagram of a general unfolded oikoplatic epithelium with its oikoplasts and fields.

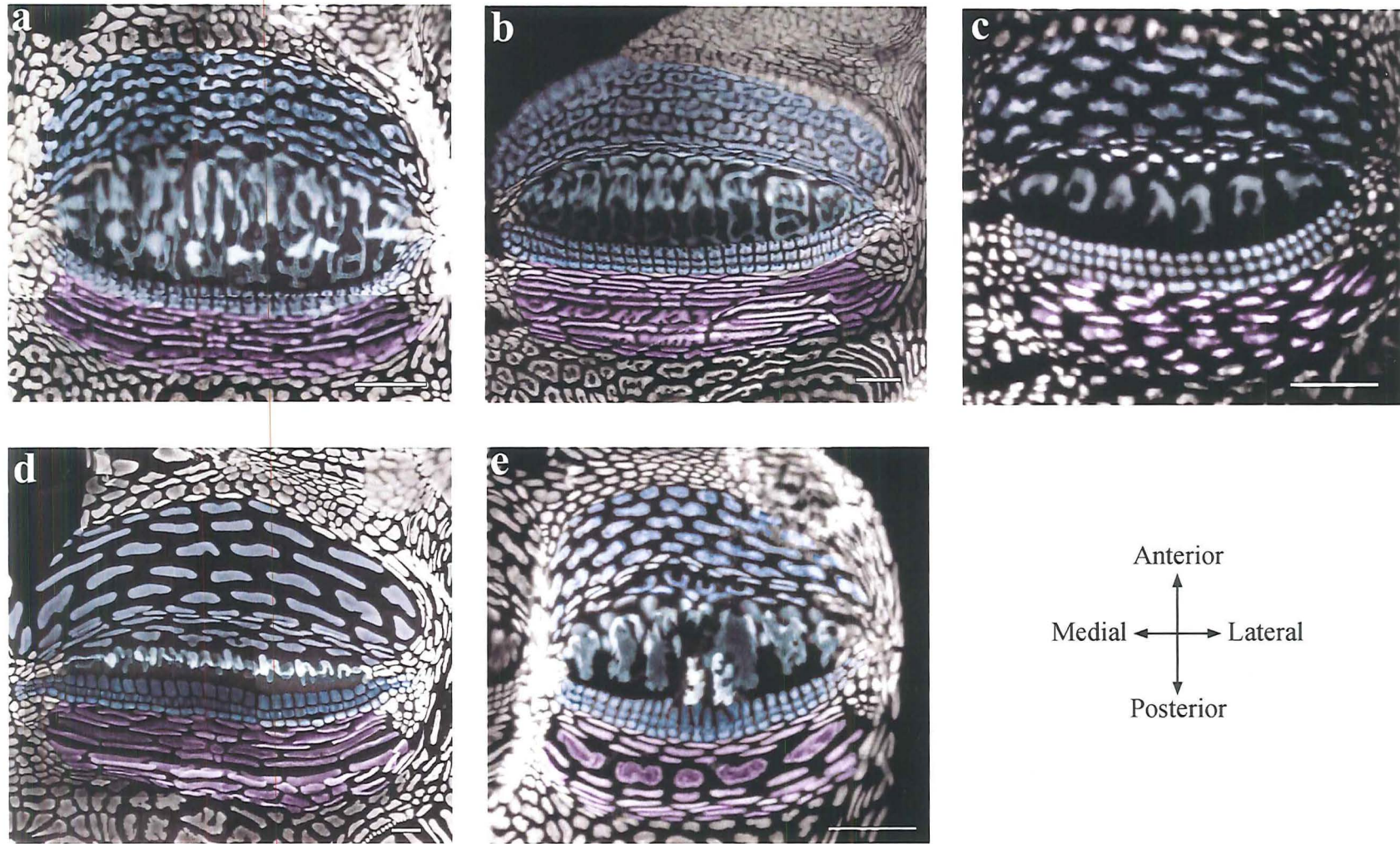


Figure 2.5
 Right oikoplast of Fol. a) *O. labradoriensis*, b) *O. vanhoeffeni*, c) *O. dioica*, d) *O. villafrancae*, e) *O. albicans*.
 Scale: 100 μ m.

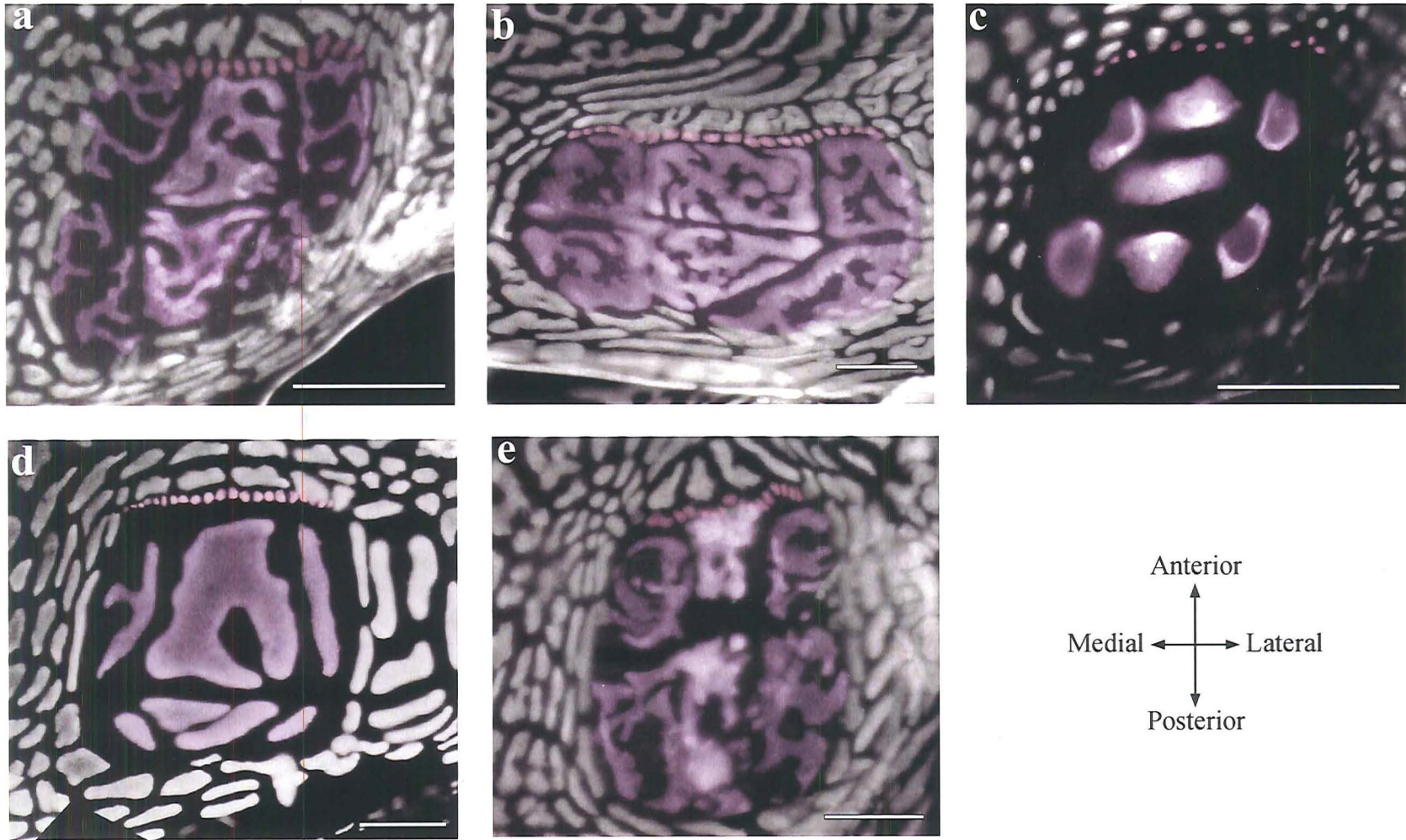


Figure 2.6
 Right oikoplast of Eisen. a) *O. labradoriensis*, b) *O. vanhoeffeni*, c) *O. dioica*, d) *O. villafrancae*, e) *O. albicans*.
 Scale 100 μm .

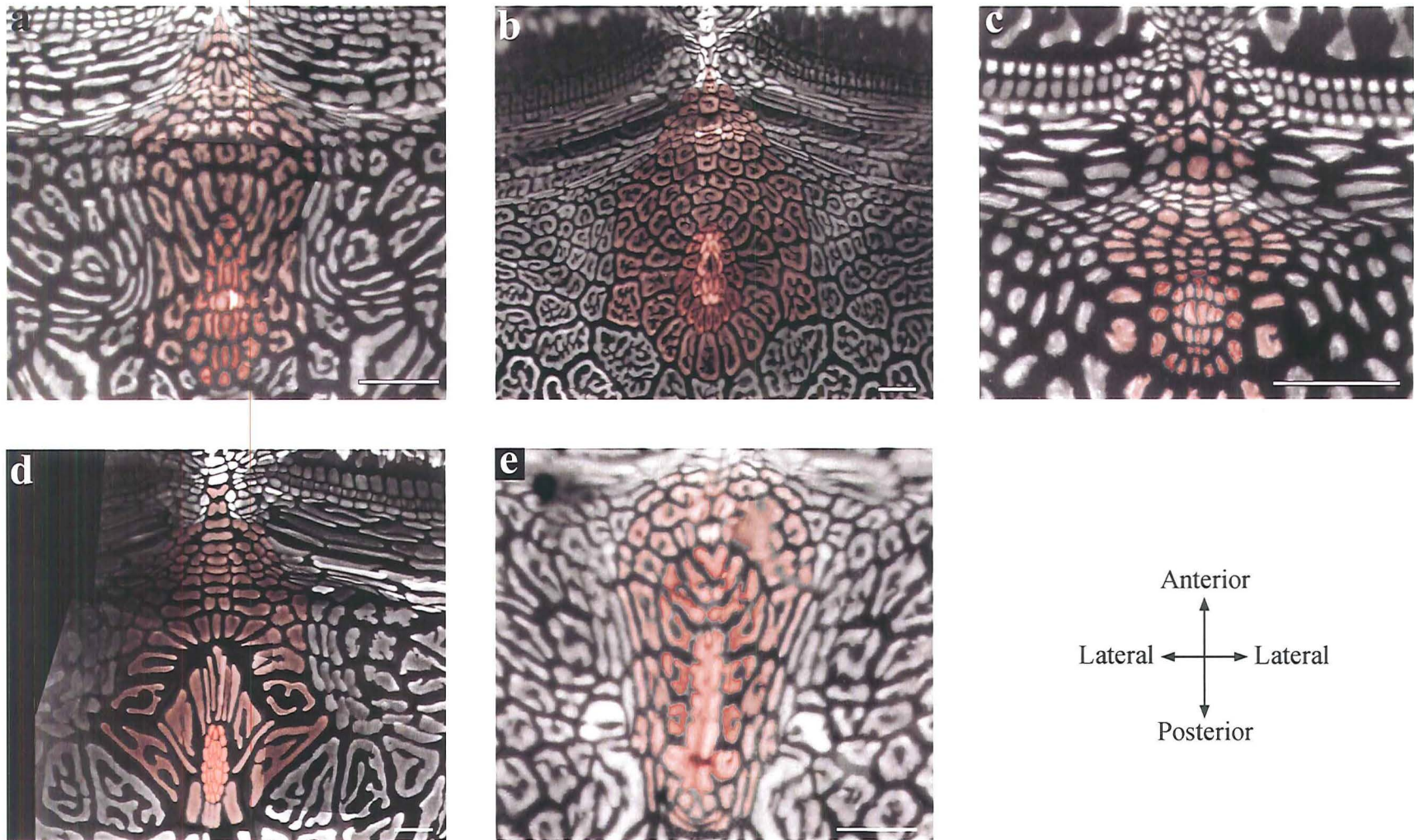


Figure 2.7
 Anterior rosette. a) *O. labradoriensis*, b) *O. vanhoeffeni*, c) *O. dioica*, d) *O. villafrancae*, e) *O. albicans*.
 Scale: 100 μm .

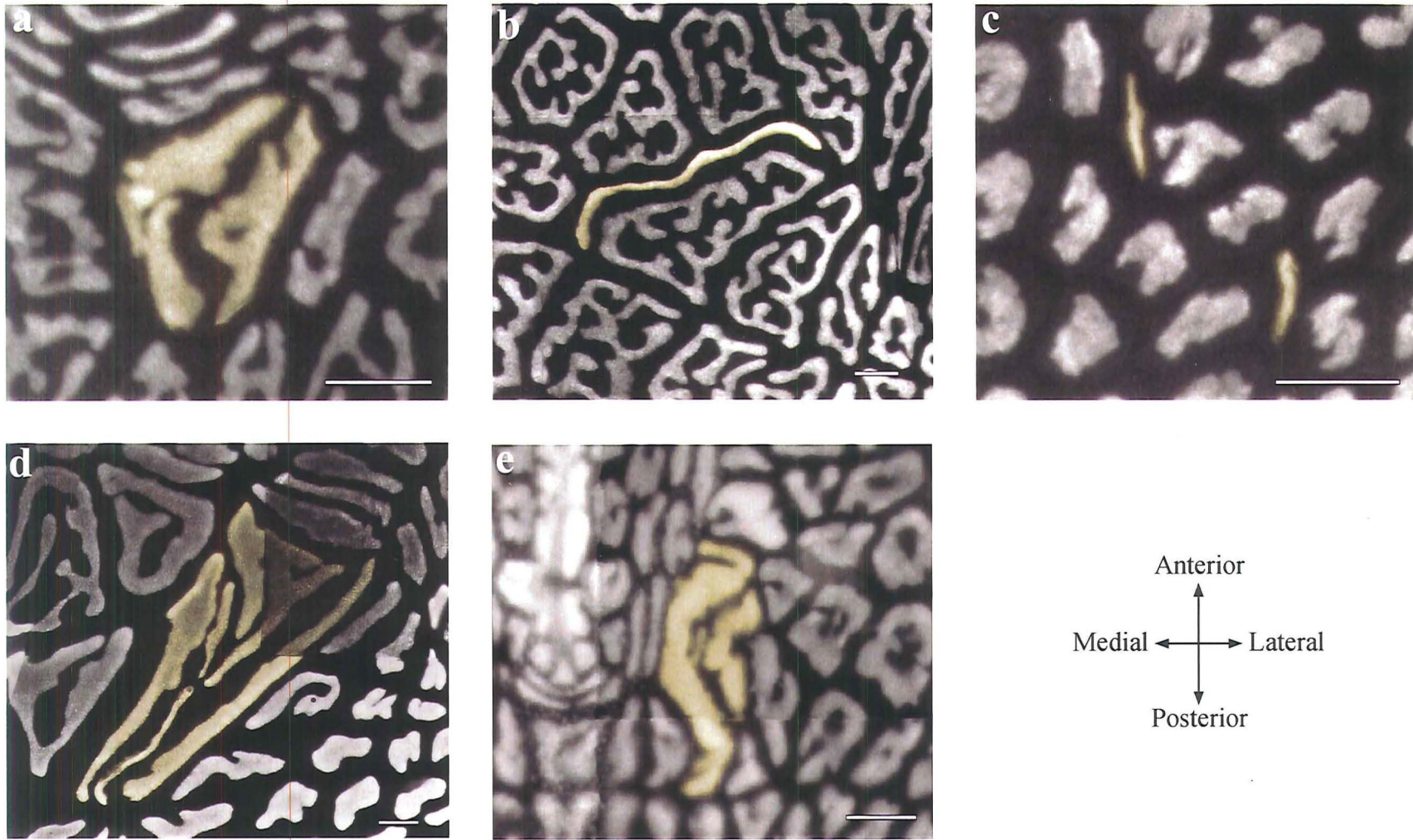


Figure 2.8

Right Leuckart cells: a) *O. labradoriensis*, b) *O. vanhoeffeni*, c) *O. dioica*, d) *O. villafrancae*, e) *O. albicans*.

Scale: 50 μm .

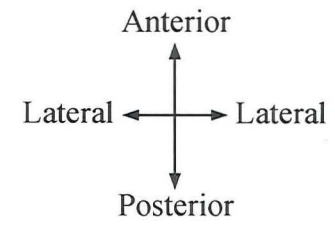
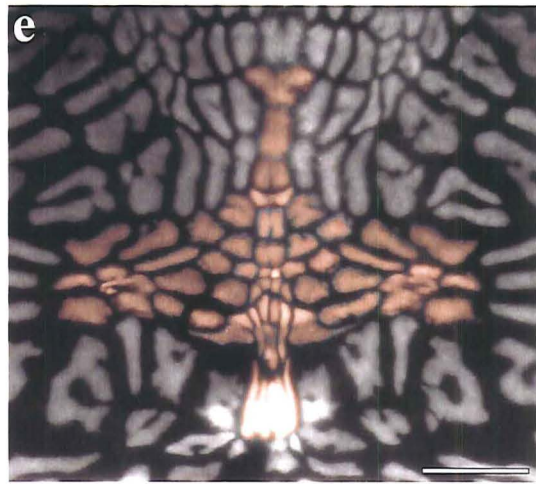
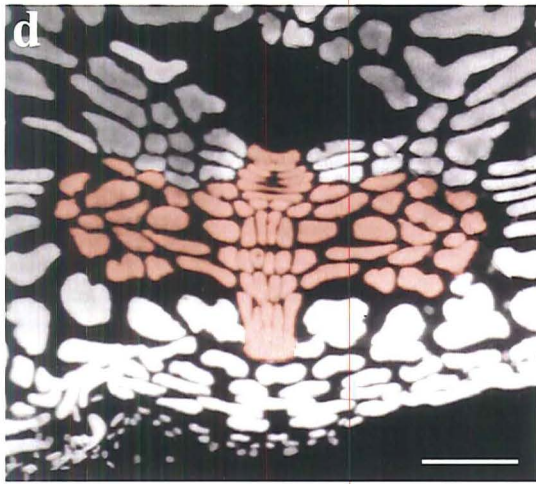
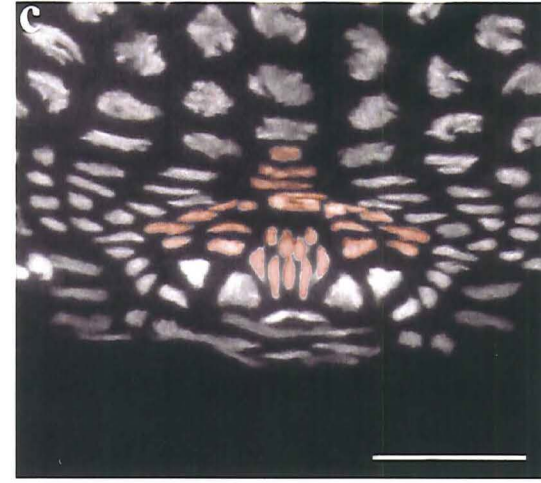
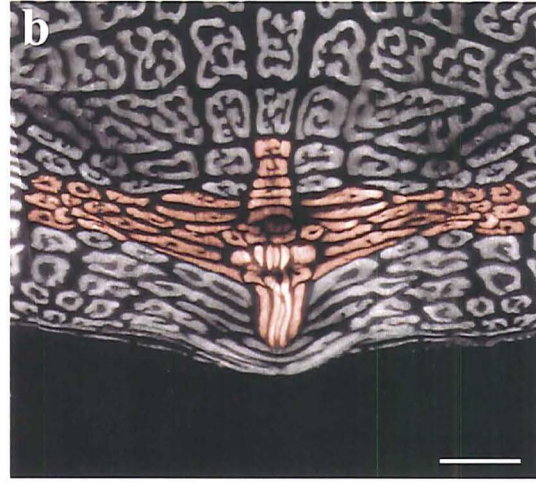
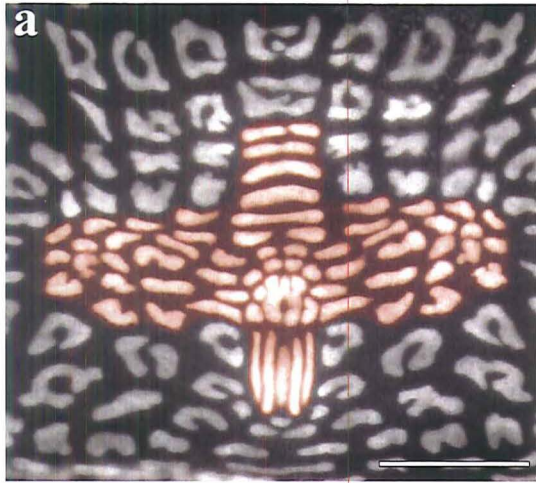
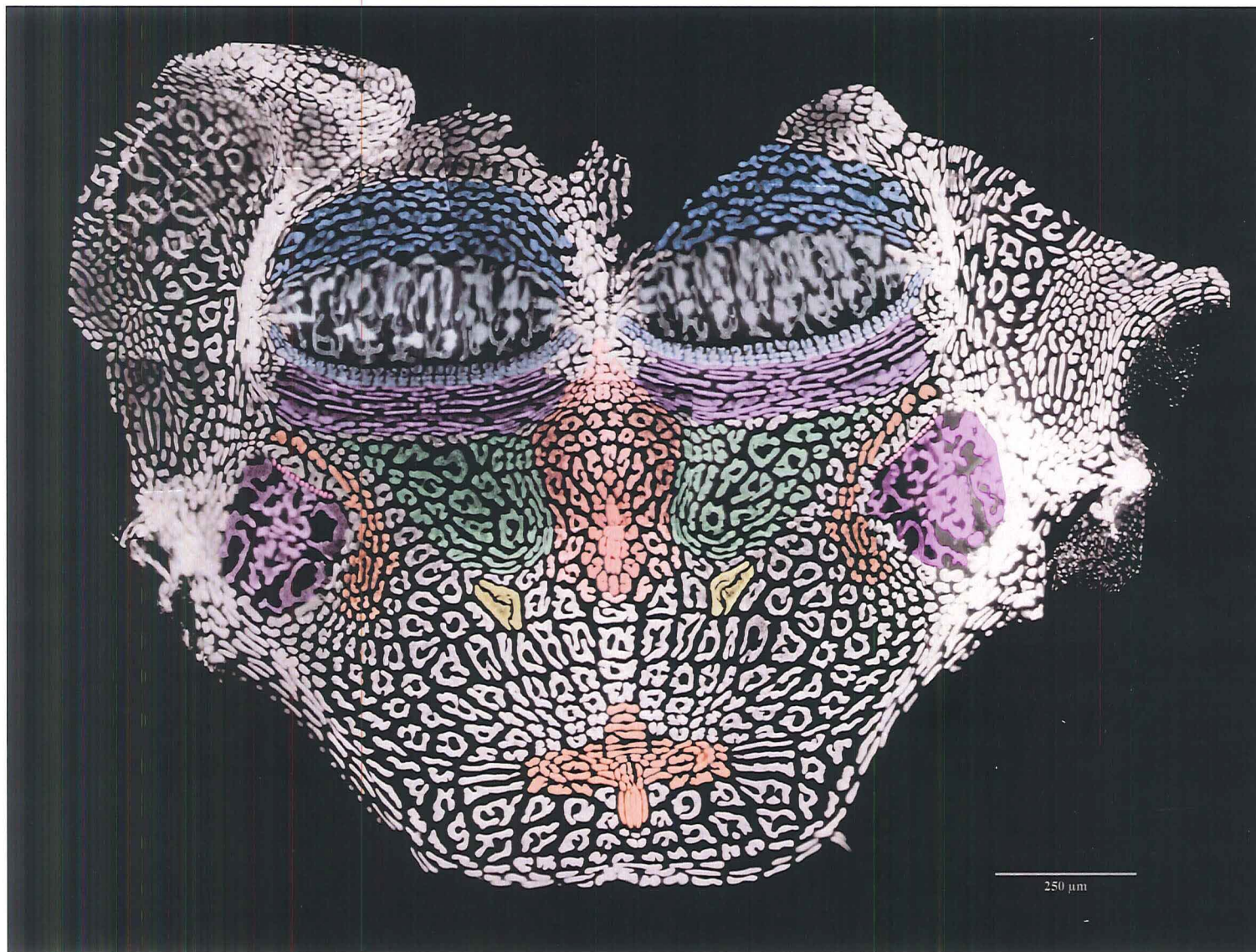


Figure 2.9
Posterior rosette. a) *O. labradoriensis*, b) *O. vanhoeffeni*, c) *O. dioica*, d) *O. villafrancae*, e) *O. albicans*.
Scale 100 μm .














-  The Fol anterior
-  The Giant cells
-  The Nasse cells
-  The Fol posterior
-  The field of Martini
-  The large cells
-  "Chain of pearls"
-  The field of Leuckart
-  The anterior rosette
-  The posterior rosette
-  The field of Ihle

Figure 2.10
The oikoplastic epithelium of *Oikopleura labradoriensis*.















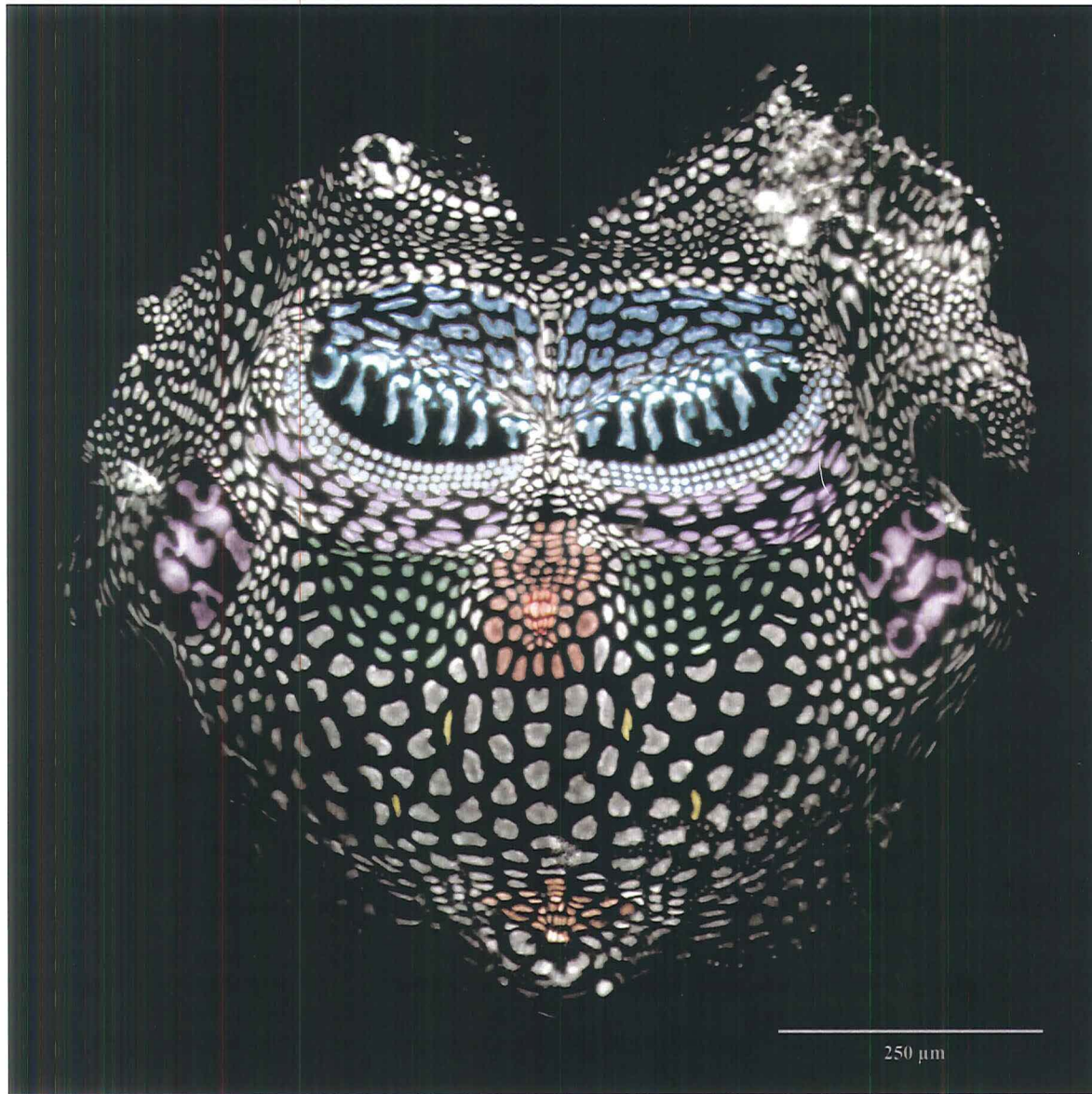
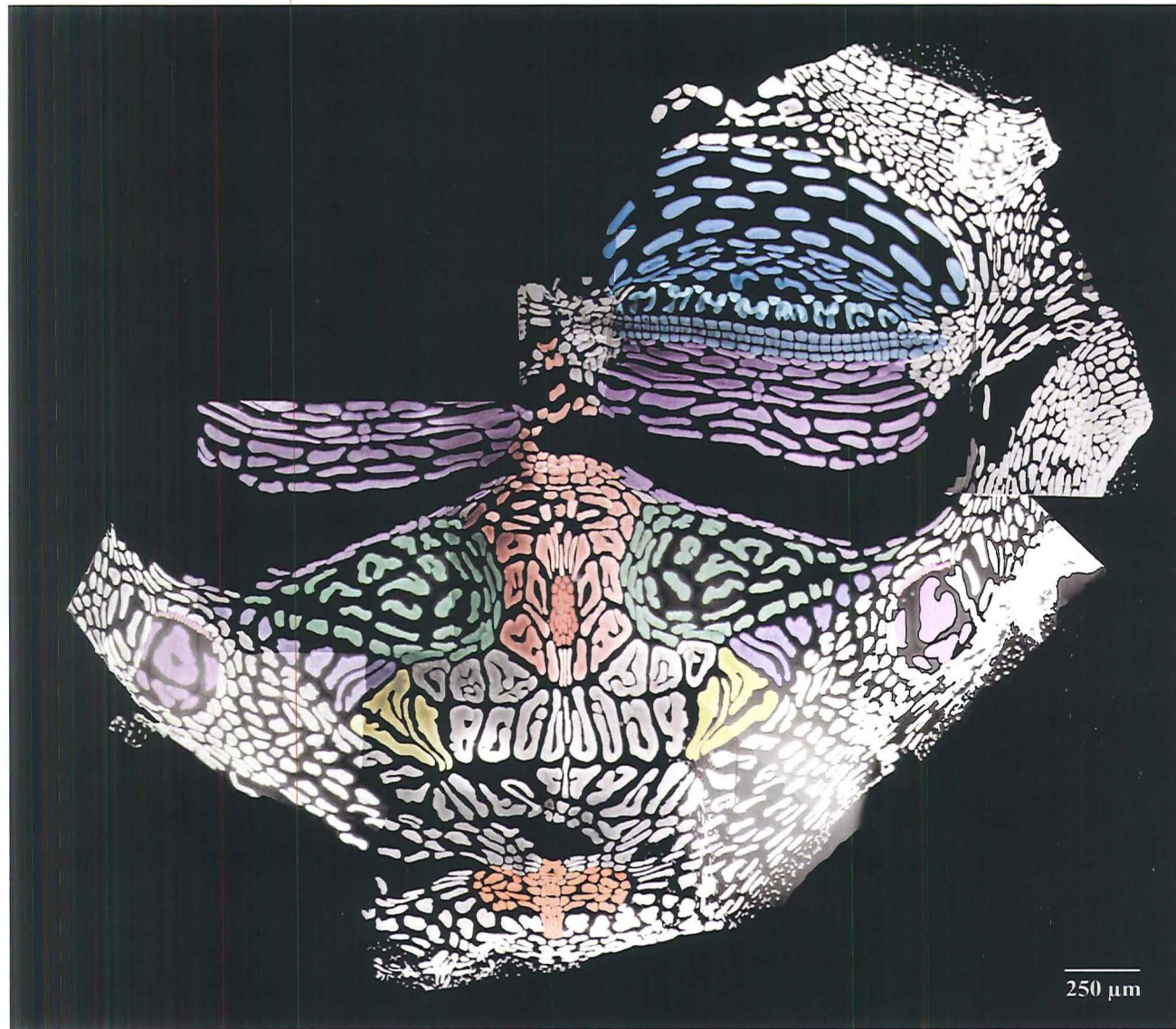
-  The Fol anterior
-  The Giant cells
-  The Nasse cells
-  The Fol posterior
-  The field of Martini
-  The oblique line
-  The large cells
-  "Chain of pearls"
-  The field of Leuckart
-  The anterior rosette
-  The posterior rosette
-  The field of Ihle

Figure 2.11
The oikoplastic epithelium of *Oikopleura vanhoeffeni*



-  The Fol anterior
-  The Giant cells
-  The Nasse cells
-  The Fol posterior
-  The field of Martini
-  The large cells
-  “Chain of pearls”
-  The field of Leuckart
-  The anterior rosette
-  The posterior rosette

Figure 2.12
The oikoplastic epithelium of *Oikopleura dioica*.














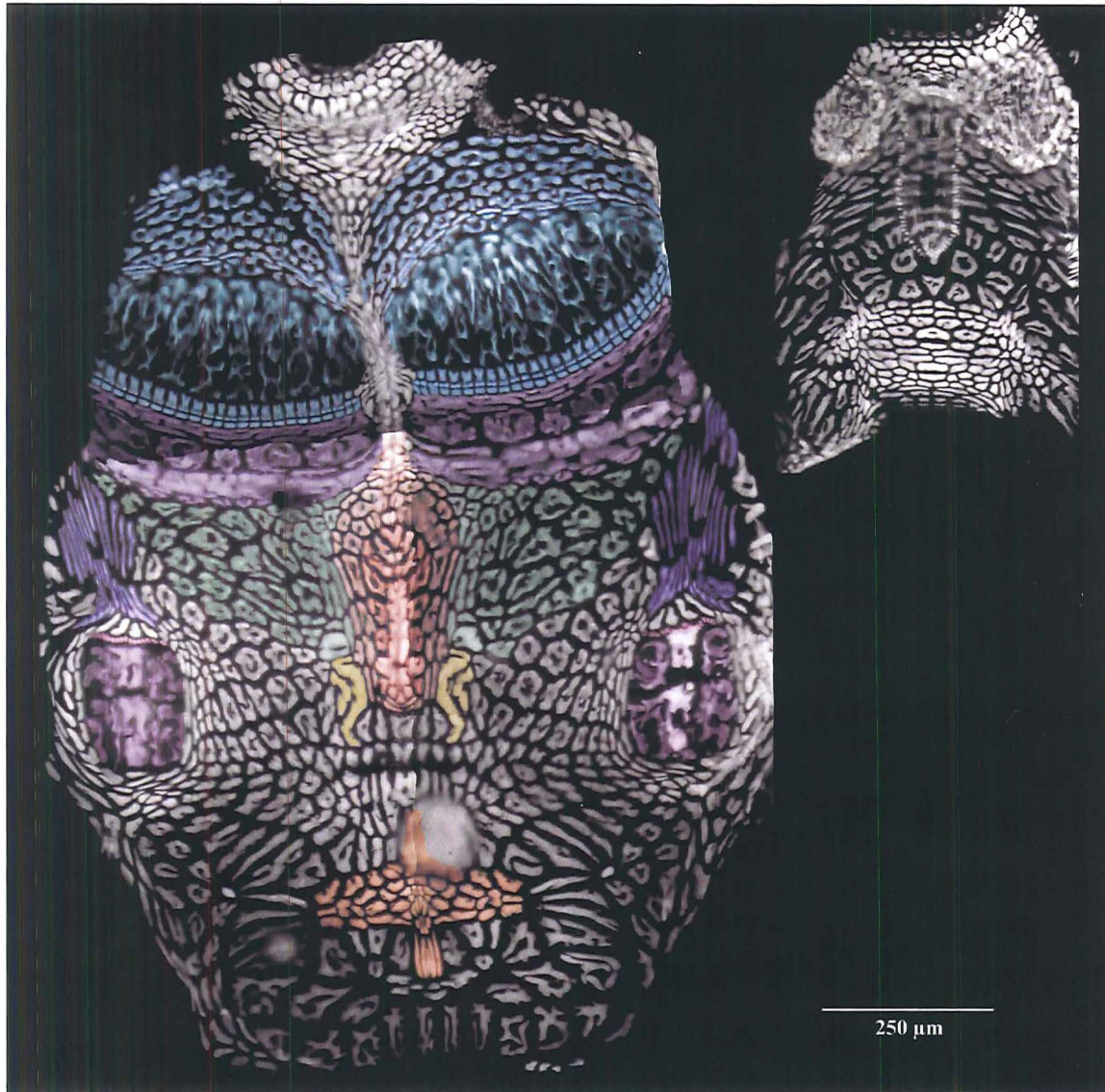
-  The Fol anterior
-  The giant cells
-  The Nasse cells
-  The Fol posterior
-  The field of Martini
-  The oblique line
-  The large cells
-  "Chain of pearls"
-  The field of Leuckart
-  The anterior rosette
-  The posterior rosette

Figure 2.13
The oikoplastic epithelium of *Oikopleura villafrancae*.





-  The Fol anterior
-  The Giant cells
-  The Nasse cells
-  The Fol posterior
-  The field of Martini
-  The large cells
-  "Chain of pearls"
-  The field of Leuckart
-  The anterior rosette
-  The posterior rosette
-  The diamond field

Figure 2.14
The oikoplastic epithelium of *Oikopleura albicans*.

	<i>O.</i> <i>LABRADORIENSIS</i>	<i>O.</i> <i>VANHOEFFENI</i>	<i>O.</i> <i>DIOICA</i>	<i>O.</i> <i>VILLAFRANCAE</i>	<i>O.</i> <i>ALBICANS</i>
<u>Oikopl. of Fol:</u>					
anterior Fol	42 cells	35 cells	62 cells	70 simple, elongated cells	55-56 cells
giant cells	8 cells	8 cells + 2 small cells at each end	7 giant cells, 2 small cells	8 cells + 2 small cells at each end	8 cells
nasse cells	32 cells	30-32 cells	32 cells	33-34 cells	26-30 cells
posterior Fol	~72 cells arranged in 10 rows	~77 cells arranged in 11 rows	~70 cells arranged in 11 rows	80 cells arranged in 11 rows	~67 cells arranged in 10 rows
<u>Oikopl. of Eisen:</u>					
large cells	2 - 2 - 2 pattern, filiform	2 - 2 - 2 pattern, filiform	2 - 3 - 2 pattern, concav/convex	2 - 3 - 2 pattern, elongated and slightly filiform	2 - 2 - 2 pattern, filiform
"Chain of pearls"	18-20 cells	20 cells	10-12 cells	22 cells	17-18 cells
<u>Anterior Rosette:</u>	total of 123 cells	total of 103 cells	total of 110 cells	total of 123 cells	total of 95 cells
centre cells	17 + 16 cells	17 + 16 cells	21 + 16 cells	17 + 16 cells	17 + 16 cells
surrounding cells	88 cells	70 cells	55 cells	88 cells	62 cells
<u>Field of Martini</u>	7 large cells. Total of 38 cells	Total of 38 cells	Total of 38 cells	7 large cells. Total of 38 cells	7 larger cells. Total of 38 cells
<u>Field of Leuckart</u>	2 interdigitated nuclei/cells	1 elongated cell and nucleus	2 elongated, but separated cells	4 cells forming a triangle	2 cells, one large elongated and one thick "L" shape.
<u>Posterior Rosette</u>	65 cells	65 cells	28 cells	65 cells	65 cells
<u>The field of Ihle</u>	27 cells, two are more elongated.	27 cells, in which 2 are more elongated.	-----	-----	-----
<u>The oblique line</u>	-----	9 cells	-----	9 cells	-----
Approximate cell number of the oikoplastic epithelium, including the ventral oikoplast.	2100	1900	1900	2600	1600

Table 2.2

Description of the regions of the oikoplastic epithelium defined in each of the five species, including the approximate cell number of the oikoplastic epithelium (including the ventral oikoplast).

The migration of the seven nuclei of the Eisen oikoplast of the larvae of *Oikopleura dioica*.

3.1 INTRODUCTION

Appendicularians (class Tunicata) are marine zooplankton organisms distributed world wide and claimed to be second in abundance only after the copepods (Bückmann, 1970). One of their most characteristic features are their secretion of disposable, mucous houses. These houses are sophisticated filtration devices for concentration of particulate and dissolved organic matter from the seawater. The filter houses are secreted by a glandular surface epithelium, the oikoplastic epithelium, covering the anterior parts of their trunks (Lohmann, 1896). The house contains two types of filters, each with a distinct role. The incurrent filter stops large and potentially harmful particles from entering the house, and the food concentrating filter retains smaller particles (down to 0,2 µm in size) (Flood, 1991).

For the last 150 years, biologists have deduced the biology of the appendicularians from studies of specimens sampled directly from the sea. Despite extensive studies, important aspects of their biology are still unknown. This is partly due to the fact that appendicularians are difficult to culture successfully in the laboratory. Only *Oikopleura dioica* has been successfully cultured (Paffenhöfer, 1973, Fenaux & Gorsky, 1979 and 1985), partly due to the fact that *O. dioica* is the only species among the appendicularians with separate sexes. This has been important in order to control the fertilization and the timing of the larval development. Because of this we chose *O. dioica* to study the development of the secretory epithelium.

Although the oikoplastic epithelium has been thoroughly studied by Lohmann (1896,1933-34) and Lohmann & Bückmann (1926), modern cell biology offers new possibilities. The epithelium of the trunk region has cells and nuclei arranged in distinct regions of different

sizes and shapes. This present paper focuses on two regions, called the oikoplast of Eisen and the oikoplast of Fol, which produce two different filters of the house (Lohmann 1933-34). The oikoplast of Eisen secretes the incurrent filter and the oikoplast of Fol produces the feeding filter. Both regions are composed of cells and nuclei that are much larger and much more convoluted than the rest of the cells in the oikoplastic epithelium (Figure 3.1).

The trunk length of an adult *Oikopleura dioica* ranges between 800 and 1400 μ m. The species is easily identified by the rounded left stomach lobe and two distinctive subchordal cells on the right side of the notochord in the distal two-thirds of the tail.

The main aims of the present study were:

1. To study the origin and differentiation of two specific regions of the oikoplastic epithelium, the Eisen oikoplast and the Fol oikoplast.
2. To study the degree of polyploidy of the Eisen oikoplast cells.
3. To investigate the shapes of the large polyploid nuclei of the Eisen oikoplast cells within the two regions, and their degree of 3-dimensional symmetry with respect to the sagittal plane.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Oikopleura dioica were collected in the bay of Villefranche-sur-Mer, France. The plankton tows were done by slow vertical tows with a wide-mouth net (mesh width of 100µm), and a 5 or 20 litre collector at the end. The tows were done in the first 20 meters below the surface. *O. dioica* were kept in non-aerated seawater at 18 degrees, in 5 litre glass jars and the animals were transferred into fresh seawater once or twice a day. The animals were kept for several generations.

3.2.2 Culture

Oikopleura dioica goes through a full generation cycle of 7-8 days at a temperature of 17-18°C (personal observations). Under natural conditions and in successful laboratory cultures, the final stages of maturation take place outside the house. The secretory epithelium and internal organs degenerate as energy is channelled into gonad production. After 5-7 days (18 °C) gametes are shed freely into the seawater before the animal dies (a few hours after spawning).

For optimal maintenance in the laboratory, animals must be suspended in gently circulating seawater, and they must be fed. We used 5 litre beakers, with gentle water circulation provided by a rotating blade, or thermal convection (Fenaux & Gorsky, 1985). The animals are very sensitive to changes in temperature and concentration of food. They require fresh seawater filtered on 50 µm nystrel net. When the particle concentration in the seawater was low, algae (*Isochrysis galbana*) filtered through 10 µm at a concentration of 10⁵ cells pr. ml. were added as additional food.

3.2.3 Fertilization

Adult animals were isolated when they reached maturity. The sexes of *O. dioica* are easily distinguished with the naked eye, since the ripe ovary is translucent and nearly colourless, and the testis is opaque and yellow. The males were collected in a beaker and kept in suspension (with a stirring bar). The females were isolated, one per petri dish (covered with 0,1% Gelatin Formaldehyde), and when the eggs were spawned, diluted sperm was added. The number of eggs produced by a single female depends on feeding conditions, temperature, and the size reached by the adult. In our cultures 150 to 350 eggs were produced per female.

3.2.4 Fixation

Larvae were fixed at different spaced developmental stages in order to recognise the oikoplast of Eisen and the oikoplast of Fol. Different fixation methods were tried. Glutaraldehyde (2% in 0,2M Na-cacodylate) introduced a high degree of autofluorescence in the tissue, so it was abandoned. Formaldehyde (3,7% in seawater) gave some background fluorescence with the chromomycin A3 stain, but was acceptable with Hoechst 33342 (Sigma, B 2261) labeling. For BrdU (5-bromo-2-deoxyuridine) fixation in methanol (-20°C) was used. This method gave the weakest background under fluorescence, but the animals were sticky and hard to microdissect.

3.2.5 DNA labeling

For DNA detection and observation of the shapes of nuclei, different fluorescent dyes were tested out. Hoechst 33342 is an A-T specific dye, which can be used for DNA labelling both in fixed and living embryos. Fixed larvae were stained 10 minutes in a dilution containing 0,5 µg/ml Hoechst 33342 in 3,7% formaldehyde (diluted in seawater) before washed in PBS and mounted in Citifluor® (Ted Pella, Ink.) (a solution of glycerol, PBS (phosphate buffer saline) and an antifading medium). *In vivo* staining was performed in 0,05 µg/ml Hoechst 33342 (diluted in seawater) in filtered seawater for about 10 minutes. The major problem with *in*

in vivo observation was that the larvae needed to be immobilised for observation of the fluorescence.

Chromomycin A3 (Sigma, C 2659), which is a G-C specific dye, (CA3) (stock 5mg/ml in ethanol) can be used on fixed juveniles (3,7% formaldehyde in seawater). Staining was performed for 10 minutes at room temperature, with a solution composed of 100 μ M CA3 diluted in PBS and 0,5M $MgCl_2$. After washing in PBS for 5 minutes the animals were mounted in Citifluor.

Mithramycin A (Aureolic acid) (Sigma, M 6891), dissolved at 100 μ M in PBS and $MgCl_2$ was found not to be as practical as Chromomycin A3. Propidium Iodide (Sigma, P 4170) is also a DNA fluorescent dye which binds to the whole DNA helix, (1 mg/ml in H_2O) and was used in a 1/50 dilution of PBS. This dye was not suitable, because it labeled both the RNA in the cytoplasm as well as the entire nuclei.

3.2.6 Immunocytochemistry (antibody labelling) of the embryo

Larvae at different stages of development were labeled with 5-bromo-2-deoxyuridine (BrdU; a thymidine analogue, 250 μ M in seawater) (Sigma, B 9285) for different period of time pulses, and the BrdU incorporated into newly replicated DNA was immunocytochemically detected with anti-BrdU antibody (Bollner, 1993, Nomura, 1991 and 1993).

Labeled larvae were fixed overnight in methanol (-20°C). The larvae were brought to room temperature, and transferred to PBS through a mixture of methanol and PBS (75/25, 50/50, 25/75 and finally 0/100). Larvae were then washed in a solution of PBS and 0,1% Triton X-100 (Sigma, T 9284), treated with 2 M HCl in PBS for 45 minutes to access DNA and BrdU, and then rinsed twice in PBS. To avoid unspecific binding during immunolabeling, 1% BSA (Bovine Serum Albumin) was dissolved in PBS. Samples were then incubated 45 minutes in anti-BrdU (Sigma) diluted 1:50 in PBS/BSA, followed by two washes in PBS/BSA. The larvae were incubated 45 minutes in anti-mouse Biotin (Sigma), followed by two rinses of PBS. Texas Red Streptavidin (Sigma, S 5138) was used as the fluorescence label (45 minutes). The larvae were then mounted in Citifluor and observed in the microscope.

3.2.7 Immobilisation of embryos

Immobilisation of the living tadpoles was performed by mounting in a viscous solution of methylcellulose (2% in sea water). The larvae were still able to move, and the dye (Hoechst) diffused out of the animal into the methyl cellulose fairly quickly. It was also tried to anaesthetise the tadpoles in MS 222 (in sea water with streptomycin/penicillin/EDTA), but the epithelium apparently degenerated after about one hour, even when the concentration of MS 222 was decreased to a minimum (1ng/ml). It was also impossible to pinch off the tail, because the body fluid then diffused out of the larvae.

The tadpoles were mounted in a drop of sea water in a special air chamber (Lutz & Inoué, 1986), but the small size of the larvae made it difficult to squeeze them adequately under the coverslip. For this reason the technique was not extensively used.

The most successful manipulation was done by mounting the larvae in a drop of sea water between slide and glass coverslip. The animal stuck to the coverglass and was then immobilised.

3.2.8 Microscopy and imaging

Live tadpoles were examined in a Zeiss IM35 microscope and imaged through a SIT video camera (Hamamatsu C2400). The image was processed through a video processing unit (Matrox card installed in a IBM PC/AT compatible personal computer and Universal imaging software: Image 1) and recorded on videodisc (Panasonic OMDR).

Epifluorescence observations were made on a Zeiss Axiophot microscope equipped with the following filters: excitation 353-377 NM, emission 395 NM; and DF (Dichroic filter) 395 NM for Hoechst; excitation 450-490 NM, emission 520-560 NM, and DF 510 NM for FITC; and excitation 450-490 NM, emission 520 NM, and DF 510 NM for Texas Red.

BrdU labeled embryos and nuclei labeled with chromomycin A3 were examined with a confocal laser scanning microscope (Leica system CLSM equipped with an Argon Krypton laser with 3 rays of excitation 488, 568 and 647 NM. Images were recorded using a slow scan.

3.3 RESULTS

3.3.1 Development of embryos

The eggs were fertilised and thoroughly rinsed with freshly filtered sea water and kept on a vibrating table at 17-18°C. Two or three minutes after fertilization, the fertilization membrane started to swell due to the cortical reaction (Holland *et al.*, 1988). Soon after, the extrusion of two polar bodies take place. The two-cell stage can be observed about 20 minutes after the sperm has been added, the four-cell stage after 25 minutes, and the eight-cell stage after 35 minutes. The first two divisions are equal, meridional while the third division occurs perpendicular to the first two. This division is slightly unequal, yielding four large cells at the animal pole (precursors of the ectoderm), and four smaller cells at the vegetal pole (precursors of mesoderm and endoderm) (Delsman, 1910). The next divisions occur about 10 minutes apart. Hatching occurs after about three hours. At this stage the larvae are motile and resemble tadpoles, which implies that the position of the tail shifts from behind the trunk to extending anteroventrally below the trunk similar to that of the adult oikopleurid appendicularians. The tail shift is by definition the «metamorphosis» of these animals (Fenaux & Hirel, 1972). The tailshift is followed by an expansion of the first mucous house produced by the animal (8h).

The mucous house now allows the animal to filter water and feed for the first time and secrete new houses. Feeding and house-building juveniles of *O. dioica* measure about 130 µm (trunk length).

3.3.2 The shape and size of the polyploid nuclei of the oikoplast epithelium

In the present study we observed that the giant nuclei of the cells of the Fol's oikoplast are curved and slightly ramified (Figure 3.2b). The nuclei of the cells of the Eisen's oikoplast have a more cup- or leaf-shaped structure (Figure 3.3).

The nuclei of the oikoplast cells of *O. dioica* are less convoluted than in other appendicularian species. The giant cells of the oikoplast of *Fol* have multilobed nuclei, while those of Eisen have nuclei that are rather leaf-shaped, convex or concave (Figure 3.3). The nuclei become more ramified with increasing age. In the rest of the epithelium the nuclei are more or less rounded and leaf-shaped (in adults).

The shape of the giant nuclei of the Eisen cells was studied by making optical sections in the confocal microscope. The optical sections of nuclei were used for 3D reconstruction in a computer to reveal their shapes. Generally the nuclei have a leaflike appearance with several cup-shaped structures (Figure 3.4).

The epithelium can be divided into two mirror images, that are bilateral symmetrical (Figure 3.1B), and stay presumably very constant throughout the postmetamorphic life span of the animal. Within these two images, cells and nuclei are formed. These have different sizes, shapes and are arranged in distinct patterns. The individual nuclei on each side of the plane are also symmetrical to each other (Figure 3.1B and Figure 3.2).

3.3.3 Differentiation of the oikoplast of Eisen

The Eisen oikoplast is composed of 7 giant cells (each about 20-30 μm in length). The cells are disposed in 3 dorso-ventral rows. The first row and the last row each contains 2 cells, while the middle row contains 3 cells. The nuclei of these 3 cells are slightly bigger and wider than the others. Anteriorly to these giant cells is a row of approximately 12 small spherical cells and nuclei, which are supposed to be the cells that secrete the inlet filters (Figure 3.1A).

The first differentiation in fixed preparations of the epithelium was observed on larvae about 6 hours after fertilization (at 17-18°C). The seven larger nuclei are at this stage easily recognised by their size, and by a more superficial location than the rest of the oikoplastic nuclei (Figure 3.5b, see arrow). About 90 minutes later, the 7 nuclei of the Eisen region have

organised into the typical 2-3-2 arrangement (Figure 3.5c, see arrow) of the nuclei that are characteristic of the oikoplast in older animals (Figure 3.5d, see arrow).

The organisation of the epithelium into defined regions takes place in the period of 6h to 7h 30 minutes after fertilization. To observe this on living embryos we used time-lapse photography. A digital photo was taken every two minutes for over two hours to observe the migration of these nuclei into their specific patterns on live tadpoles. Figure 3.6a-c shows the results from time-lapse recorded images from a live tadpole in the period from 6h to 7h after fertilization. Figure 3.6d-e shows the Eisen oikoplast from fixed tadpoles of corresponding ages. These observations show that the cells and the nuclei arranged themselves in the characteristic 2-3-2 pattern within 60-90 minutes.

3.3.4 Differentiation of the oikoplast of Fol

The oikoplast of Fol is composed of four different regions: the Fol anterior, the giant cells, the three rows of small spherical nuclei (the Nasse cells) and the Fol posterior.

The Fol anterior can be observed as invaginations of the epithelium at approximately 6h after fertilization, and their nuclei seem to be larger than the average epithelial nuclei. We can easily distinguish three regions (Figure 3.5 a, b and c). Region **a** develops into the posterior Fol (Figure 3.2d). Region **b** develops into the giant cells and the three organised rows of the Nasse cells (Figure 3.2 a and b). Region **c** will probably form the anterior Fol, which is located in front of the giant cells (Figure 3.2c).

3.3.5 DNA replication

Labelling of embryos with short pulses of BrdU (3-15 min.) allows visualisation of DNA replication. We observed that nuclei in specific regions of the epithelium at a given time replicated, while those in other regions did not. Figure 3.7 shows three different tadpoles 7 hours after fertilization. Our results indicate that **a** and **b** can be parts of the oikoplast of Fol and region **c** can be identified as the region called the posterior-dorsal field (Fenaux 1971). It

consists of cells with large nuclei. The region of Eisen was labeled, but only 6 nuclei could be counted although the region was expected to contain 7 nuclei.

Double labelling with BrdU and specific DNA dyes (Hoechst 33342, Chromomycin A3, Propidium iodide) was not successful, due to the exposure of samples to HCl (to make DNA accessible) so BrdU positive nuclei could not be precisely identified with respect to position within regions. However, the oikoplast of Fol appears unlabeled at 7 hours of development.

3.4 DISCUSSION

The shapes of the epithelial nuclei and cells vary between species of appendicularians. They also vary with the age and size of the specimen and with the different regions of the oikoplast. Most nuclei in adult appendicularia are large and ramified. This provides a large area of contact between nucleus and cytoplasm. This may accelerate the exchange between nucleus and cytoplasm and help carry out the messenger RNAs necessary for high secretory activity of these cells.

The increased nuclear volume of an appendicularian is clearly due to the existence of polyploidy. It has been suggested that the degree of polyploidy reside only in the fact that the chromatin in the larger nuclei is more scattered, and that the chromatin in the smaller nuclei have less space and is therefore more compact. By comparing the chromatin in oikoplast cells and in the spermatozoid, Fenaux (1971) showed that the degree of polyploidy of the nuclei is very high (in *O. albicans*, measured to 1024 n) for the giant cells of the region of Fol and it is generally very variable from one region to another. Fenaux (1971) also showed that the degree of polyploidy is proportional to the size of the animal. It would thus be of importance to find the exact degree of polyploidy in these cells. It has been observed in the present study that the shapes and forms of the nuclei change with the age and that the degree of polyploidy increases with the age of the animal.

This study describes in particular the differentiation of the secreting epithelium and in particular the Eisen oikoplast in *Oikopleura dioica* during early development. The seven larger cells and nuclei of the Eisen region can first be identified 6 hours after fertilization. The nuclei are arranged in a characteristic manner, and the nuclei/cells migrate into their final position only 7h to 7h 30 after fertilization.

Presumably the nuclei position themselves as a result of cell migrations into the specific 2-3-2 pattern formed in the adult animals but we have not observed the cell's boundaries yet.

The observations of epithelial cell nuclei labeled with BrdU showed that nuclear DNA in specific regions of the DNA was replicating, while in other regions it was not. These switching on and off of DNA replication suggests that cells of a specific region are replicating their DNA synchronously. Either the cells in these regions originate from the same precursor cell(s) early in the embryological development and continue to divide and replicate synchronously, or specific neighbouring cells are connected in a syncytium. These questions should be further investigated with the use of electron microscope in order to develop the cell lineage for the *Oikopleura dioica*.

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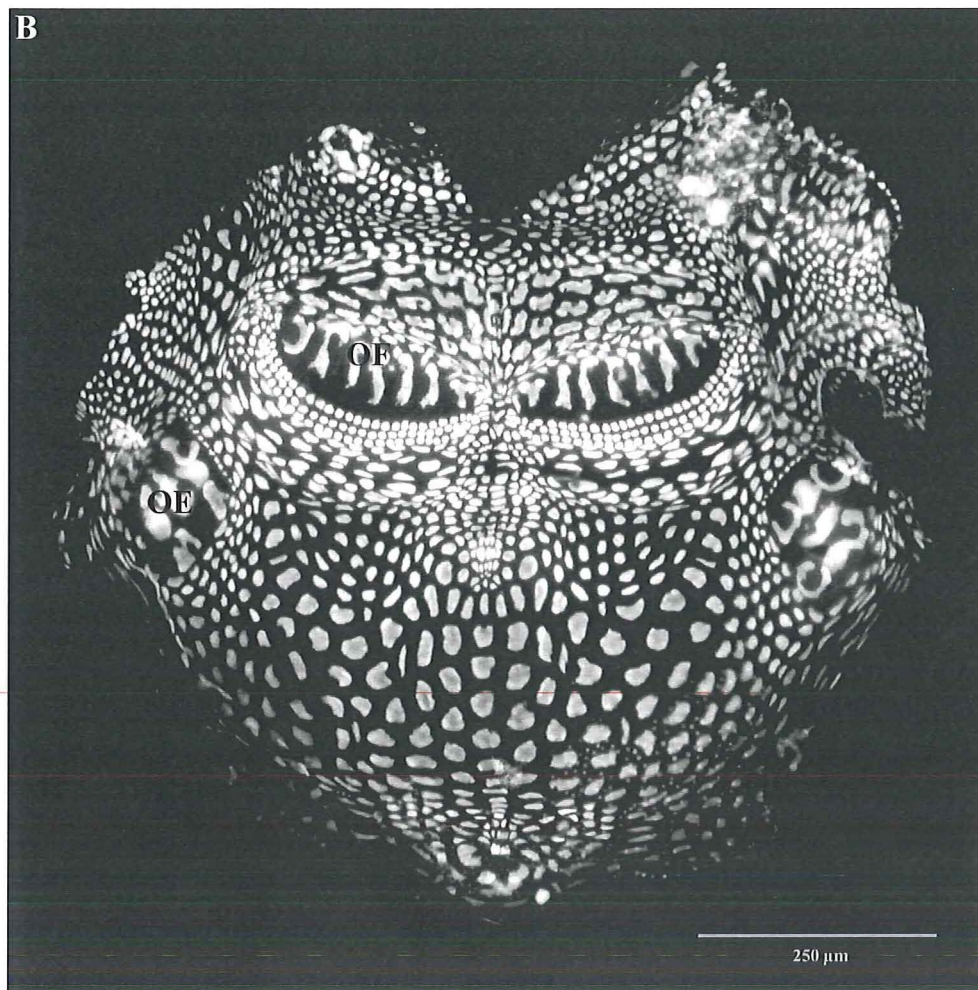
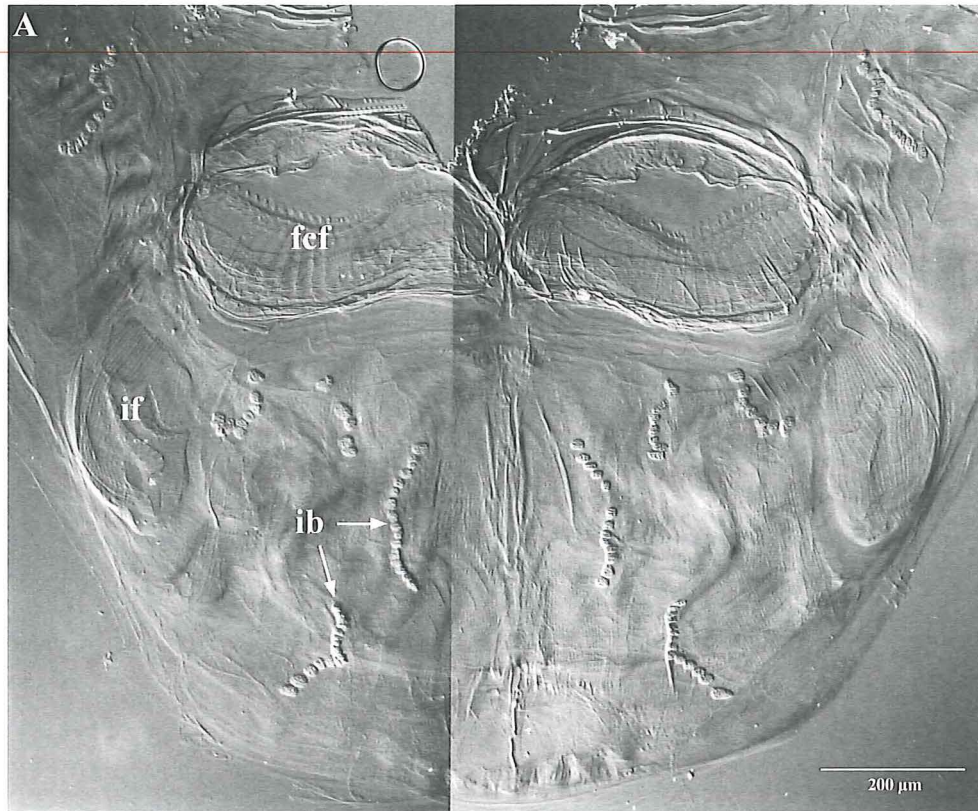


Figure 3.1
 A) House rudiment and inclusion bodies (ib) of *Oikopleura dioica*. fcf: food concentrating filter, if: inlet filter. B) The unfolded oikoplastic epithelium of *Oikopleura dioica*, labeled with Hoechst. OE: oikoplast of Eisen, OF: oikoplast of Fol.

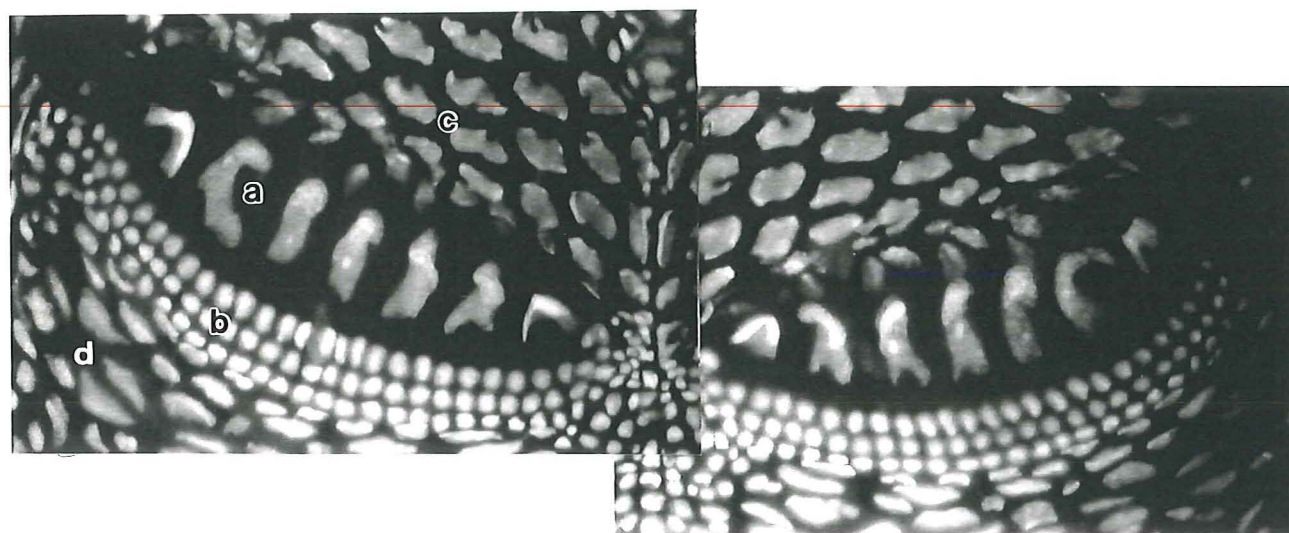


Figure 3.2

The oikoplast of Fol consist of four distinctive regions. In *Oikopleura dioica* there are seven giant nuclei/cells (b) which are convoluted and ramified. Posterior to the giant nuclei we find three distinctive rows of small spherical nuclei (a), called the Nasse cells. Posterior to these there is a region of elongated leaf-shaped cells, called the posterior Fol (d). Anterior to the giant nuclei we find a similar region with flat nuclei, called the anterior Fol (c).

Enlargement 46 times.

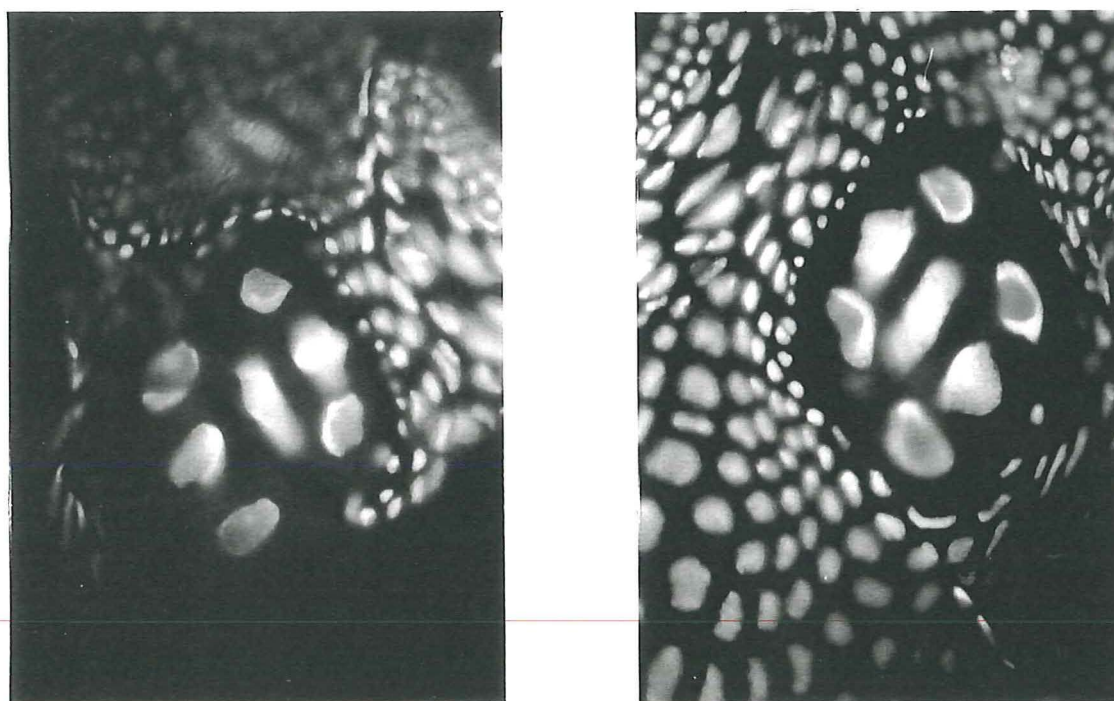


Figure 3.3

The oikoplast of Eisen consist of seven large nuclei arranged in a 2-3-2 pattern. In this animal (*O.dioica*) the three nuclei in the middle are convex, while the four other nuclei are concave. Aterio-lateral to these cells we find a ribbon of approximately 12 nearly spherical nuclei. These cells are supposed to secrete the inlet filters.

Enlargement 46 times.

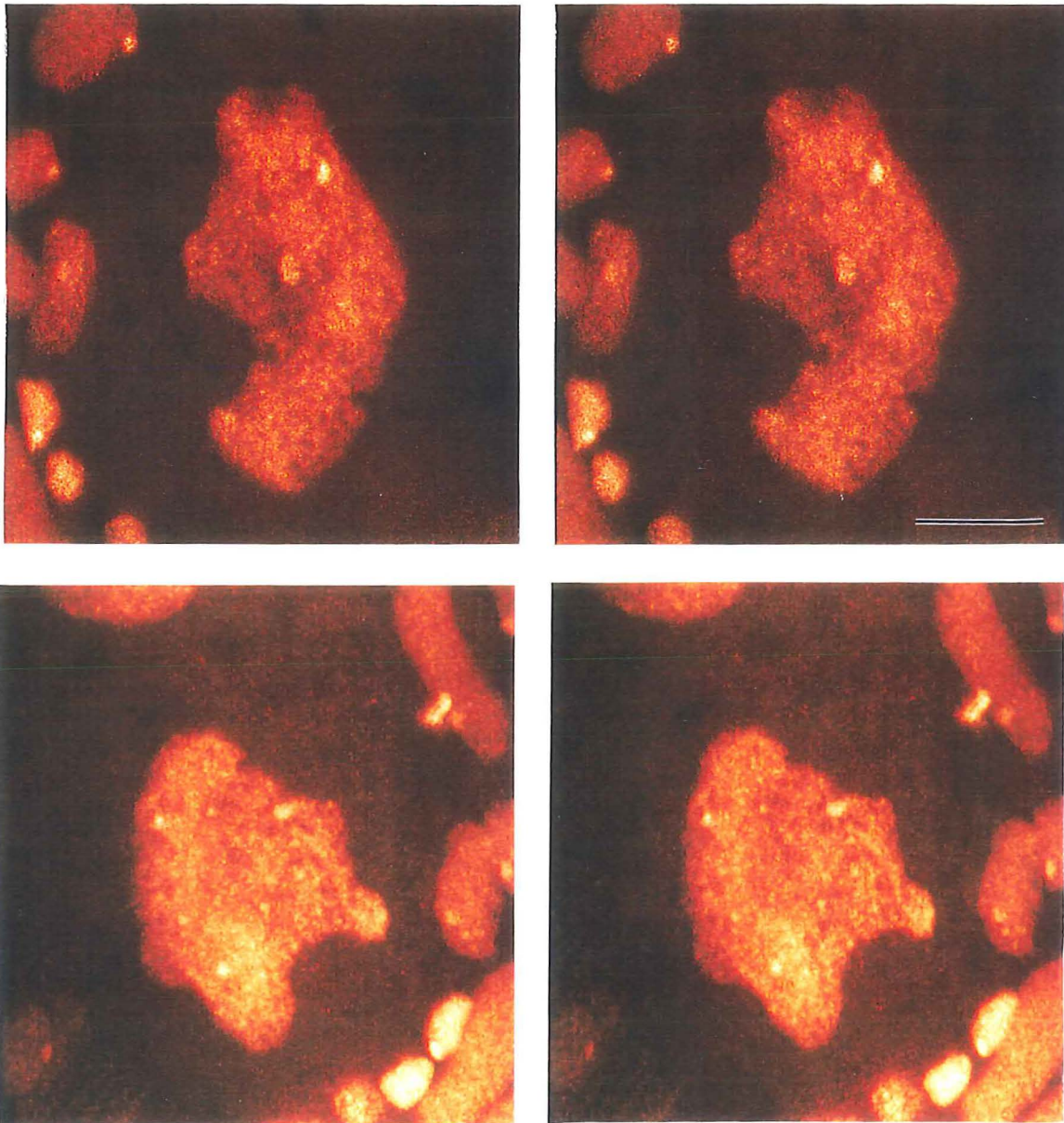


Figure 3.4

Stereo pairs of 3-D reconstruction of optical sections collected through the depth of two nuclei of the oikoplast of Eisen. They reveal the typical leaflike three-dimensional character. These nuclei are taken from an adult *Oikopleura dioica* and represent the same nuclei from the contralateral regions of Eisen. The nucleoli are visible as brighter dots in the middle of the nuclei.

Scale: 10 μm .

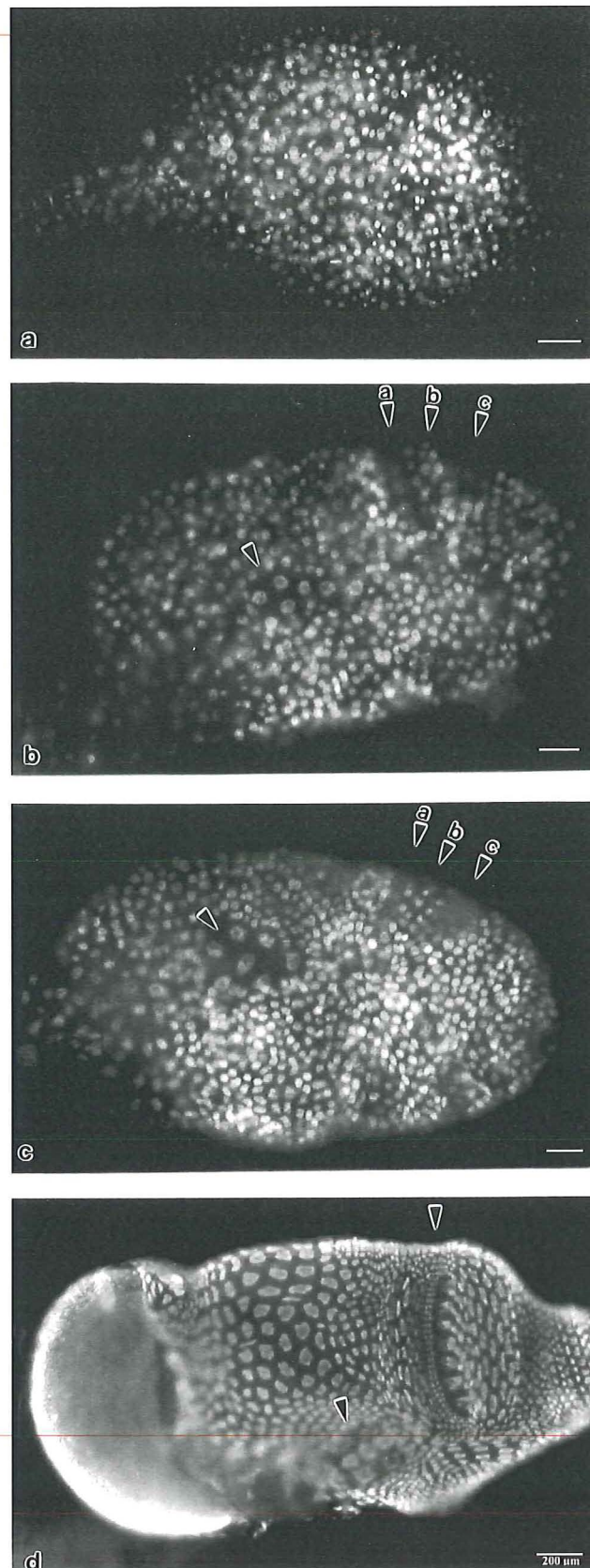


Figure 3.5

Tadpoles were fixed at different stages of development and labeled with Hoechst 33342. (a) shows us the epithelium of a tadpole four hours after fertilization with no sign of differentiation. The nuclei have all the same size and there do not seem to be any sign of organization of the epithelium into distinct regions. The oikoplast of Eisen (arrow) is first observed in a tadpole about six hours after fertilization (b). The nuclei are at that time arranged in a specific manner and it's not until the tadpole reaches seven and a half hours (c) that the nuclei are arranged in the typical 2-3-2 arrangement. Figure 3.5 d shows an adult appendicularian with the typical mosaic epithelium. Scale: 25 μm, except for (d).

Time-lapse

Fixed stages

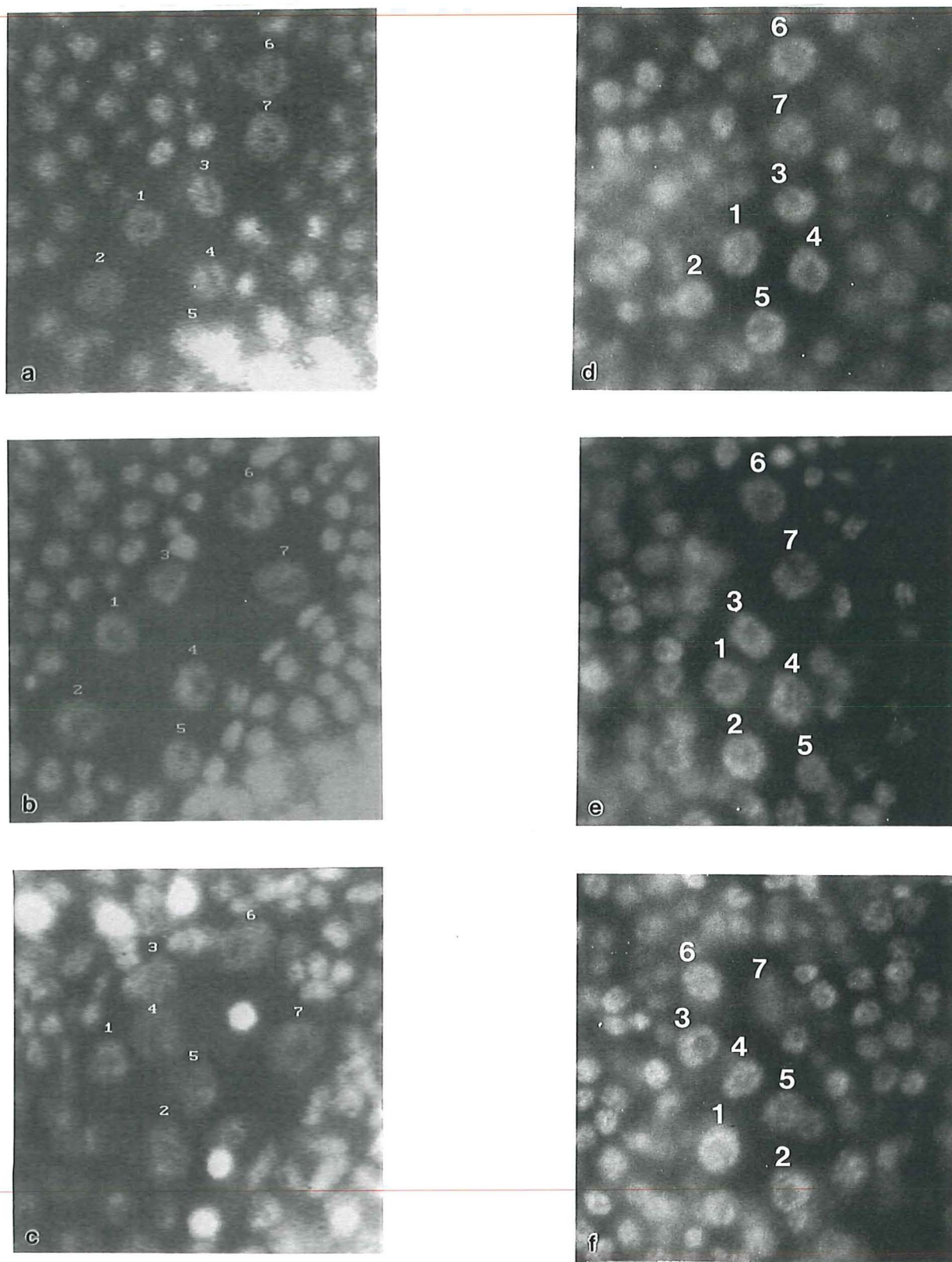


Figure 3.6

The seven nuclei of the region of Eisen and their migration to final position. (a-c) show time-lapse images of a live tadpole. (a) is taken about six hours after fertilization, (b) about six and a half hours and (c) about seven hours after fertilization. (d-f) are from fixed tadpoles. (d) corresponds to six hours, (e) to six and a half hours and (f) corresponds to seven hours after fertilization. These complementary observations i.e. the nuclei (and presumably the cells) arranged themselves in the characteristic 2-3-2 pattern within 60-90 minutes.

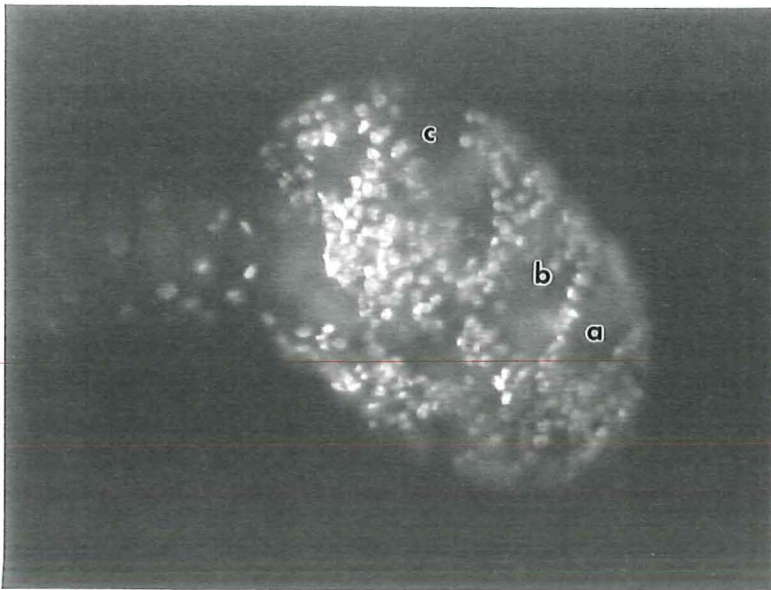
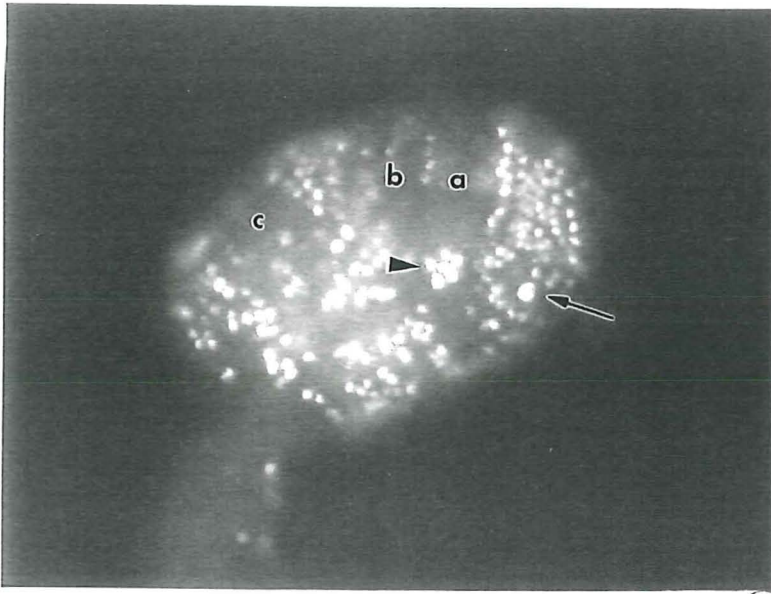


Figure 3.7
BrdU was incorporated (15 minute puls) into duplicating DNA strands in a seven hour old tadpole and detected immunocytologically with a specific monoclonal antibody. The pattern of incorporation indicates that some regions of the epithelium DNA replicates, while others do not. These three different tadpoles are about seven hours old and display three particular regions (a, b and c) that seem to be "switched" off at the time of BrdU incorporation.