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External morphology of the eggs of *Asplanchnopus multiceps* (Schrank, 1793) (Rotifera): solving the 150-year-old case of mistaken identity

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External Morphology of the Eggs of *Asplanchnopus multiceps* (Schrank, 1793; Rotifera): Solving the 150 year-old case of mistaken identity.

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Abstract

This report calls into question the practice of passing along illustrations and anatomical descriptions from the literature without scrutiny. An error made by Leydig (1854) in characterizing the egg of *Asplanchnopus multiceps* was perpetuated in authoritative publications (Hyman, 1951; Voigt, 1957; Ruttner-Kolisko, 1974; Koste, 1978, 1987) well into the 20th century. Daily tracking of individual mictic and amictic female *A. multiceps* demonstrates that the structure formerly considered to be a diapausing egg is, in fact, subitaneous. It develops without arrest into a male or female rotifer. True resting eggs containing dormant embryos are characterized by a very dark interior. In the majority of these eggs the interior is surrounded by a halo-like zone consisting of a clear space and an external layer. The correct identification of subitaneous and resting eggs in *A. multiceps* confirms the description of Plate (1885) and firmly establishes the mode of reproduction of this species as oviparous. Mictic females lay haploid male eggs first, followed by resting eggs if fertilization has taken place. In the absence of fertilization, mictic females lay significantly more (male) eggs than amictic females lay female eggs; however, their lifespan does not differ markedly.

Key words

Oviparous, ovoviparous, subitaneous egg, resting egg, amictic, mictic

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Introduction

Asplanchnopus multiceps is a large, predatory rotifer that favors the littoral zones of oligotrophic lakes and rivers. Unlike other members of the Aplanchnidae it is oviparous (Ruttner-Kolisko, 1974; Nandini & Sarma, 2005). Amictic females produce eggs that develop immediately upon laying into diploid females, while mictic females produce haploid ovocytes that develop into males if they are not fertilized or into diploid resting eggs that enclose a dormant embryo if they are fertilized. Resting eggs are capable of withstanding unfavorable environmental conditions and can survive over a prolonged period of time. (Gilbert, 1974; Pourriot & Snell, 1983). They are a source of genetic variability and aid species dispersal (King, 1980). Their external morphology is distinctive enough that, when combined with other features, it can be used to distinguish between closely related species (Gilbert & Wurdak, 1978). Several publications describe shell deposition (Mràzek, 1897; Tannreuther, 1920; Bogoslovsky, 1960; Wurdak et al. 1977) and early embryonic development of resting eggs in oviparous (Bogoslovsky, 1929; Nipkow, 1958; de Beauchamp, 1956) and ovoviviparous (Nachtwey, 1925; Lechner, 1966) monogonont rotifer species. However, the internal organization of the resting-egg embryo is not fully understood and significant questions remain regarding the resumption of development prior to hatching (Gilbert, 1988).

The current report is part of a broader study on embryonic development in rotifers with particular emphasis on resting eggs. In previous publications the resting egg of *A. multiceps* was presented as a sphere bearing short filaments on its surface (Ruttner-Kolisko, 1974; Koste, 1978). This communication demonstrates that this fuzzy egg is, in fact, subitaneous and presents images obtained through light and scanning electron microscopy of both subitaneous and resting eggs of *A. multiceps*. The mischaracterization of the mode of reproduction and of the structure of the resting egg is traced to the earliest reports. The possibility is raised that similar errors may exist in the description of other rotifers leading to problems in identifying closely related and cryptic species.

Subitaneous and resting eggs of *A. multiceps* are convenient objects for experimentation due to their large size, abundance, easy collection and handling. The development of both subitaneous and diapausing egg embryos is external in this species, whereas in *Asplanchna* the two types of embryos develop in different environments. Our understanding of resting egg ultrastructure and internal organization in the Rotifera is very limited. Contributions in this area can address larger questions related to the formation and composition of external coverings, yolk and pigment, as well as, the functions of various egg components in protection, dormancy and development.

Materials and Methods

Adult *Asplanchnopus multiceps* females were isolated from Lake Sagatagan in Collegeville, Minnesota during the summer of 2015. They were cultured under continuous light at 21^0C on a diet of other rotifers, principally *Philodina*. *Philodina* sp. were collected from local birdbaths and maintained on an algal diet. *A. multiceps* eggs were gently removed from the bottom and sides of the culture vessel using an Irwin loop and transferred to a slide. Egg diameter was measured under a light microscope at 100x magnification. Live eggs and rotifers were photographed using the Leica LasEZ system. Eggs and neonates were placed into individual petri dishes to permit daily observation.

For scanning electron microscopy, eggs were rinsed 3 times in spring water. Some eggs were treated with 2% bleach to remove adhering bacteria and rinsed again. Eggs were fixed in 2% glutaraldehyde in .05M cacodylate buffer, pH 7.4, overnight at room temperature. The next day they were rinsed in 3 changes of .1M cacodylate buffer and postfixed in 1% osmium tetroxide in .1M cacodylate buffer at 4^0C. They were dehydrated in alcohol, critical point dried, sputter coated with gold and observed under a Hitachi S530 scanning electron microscope operated at 5 – 15kV. Images were captured and processed using the QuartzPCI software.

Results

Figures 1 and 2 illustrate a live *A. multiceps* amictic female and male respectively. *A. multiceps* males are roughly 2/3 the size of the females (fig. 2). Figure 3 shows a mictic female carrying a developing ovocyte posterior to the stomach. There are no obvious differences between amictic and mictic females in body size or shape. Their size varies with age. Mictic females can be distinguished from amictic females only through observation of the eggs they produce. Male eggs can be recognized by their smaller size. Until they are inseminated, mictic females produce eggs that hatch into males. Once copulation takes place the female produces only resting eggs and will not lay any more male eggs. Consequently, the number of male eggs produced by a given female depends on how soon she is mated. If the male eggs are removed each day prior to hatching the mother will continue to lay haploid male eggs until death. If a male offspring is permitted to hatch in the same culture dish as its female parent he will inseminate her and initiate the development of resting eggs. The number of resting eggs a given female produces depends on diet and on her age at first successful copulation with a male. Typically, a female will lay one or two resting eggs in her lifetime, but five resting eggs were counted in one case. In the confines of the culture dish *A. multiceps* females will cannibalize conspecific males, including their own offspring and those they just mated with.

Developing subitaneous ovocytes have medium grey contents, whereas developing resting eggs, along with the adjoining portions of the vitellarium are very dark. At maturity the egg volume is about the same as the volume of the full stomach. Ovocytes remain flexible until late development. One to two hours prior to being laid the shell hardens, the ovocyte becomes visibly spherical and, in the case of subitaneous eggs, covered with a short fuzzy external layer. All eggs are firmly attached to the bottom and sides of the culture dish at the time of laying. Three classes of eggs were observed:

1. Large eggs with a fuzzy outer wall having an average diameter of 249 μm (N = 27, SD = 10.9), and moderately dense contents (Fig. 4). These eggs are diploid; they develop into amictic or mictic females immediately after deposition.

   The egg shown in figure 4 is freshly-laid and alive. Beneath the fuzzy shell there is a peripheral zone containing large and small clear vacuoles. These vacuoles disappear when development commences.
2. Small fuzzy eggs measuring 192 μm in average diameter (N = 27, SD = 19.5) shown in figure 5. These are unfertilized, haploid eggs that develop into males. The egg in figure 5 is in the four-cell stage. There is one large cell and 3 smaller cells. This configuration is very similar to developing embryos in the ovoviviparous *Asplanchna girodi* Guerne, 1888 (Lechner, 1966). Figure 6 illustrates the empty shell left behind by a male hatchling. The shell opens into two nearly equal halves. Furrows marking the line of separation between these halves are not externally detectable in intact eggs.
3. Large, very dark eggs whose average diameter is 259 μm (N = 28, SD = 9.32). These eggs, illustrated in figure 7, contain diploid dormant embryos. The egg is nearly perfectly

spherical. There is a clear zone between the outer shell layer and the very dense interior in the egg illustrated. The outer wall bears very short fibrils. In some instances, however, the clear zone is narrower or missing between the outer wall and the interior and the outer perimeter is smooth. These eggs are presumed to be at an earlier developmental stage.

The differences between subitaneous and resting eggs are more evident under the scanning electron microscope. The fuzzy exterior of the wall of the subitanous egg of the larger variety can be seen in figure 8. Mild bleach treatment fails to remove pieces of debris stuck to the filaments. Figure 9 shows the circular adhesive patch that anchors the egg to the substrate. Figure 10 is a high magnification image of the non-adhesive portion of the surface where filaments predominate. Filaments measure around 12 µm in length and splay out into multiple rootlets at the point of attachment. Judging by the variety of their configurations, the filaments are flexible. Between filaments the surface is smooth and clean. Figure 11 is an image of the resting egg taken at the same magnification as the subitaneous egg shown in figure 8. The wall lacks an external layer of filaments altogether and appears somewhat porous. Only a portion of the adhesive patch was preserved. The high magnification view in figure 12 shows both the adhesive patch and the general surface. The adhesive patch is smooth, while the general surface is marked by short microvilli-like projections and minute pores.

A comparison of the fecundity and longevity of amictic and mictic females yielded the following data. Amictic females laid an average of 6.85 eggs (N = 27, SD = 3.19). The number of eggs laid by a single female ranged from 1 to 15. From the egg stage until death their mean lifespan was 8.85 days (SD = 1.03). Mictic females laid an average of 11.5 male eggs (N = 12, SD = 4.68). The number of subitaneous eggs laid by a single female ranged from 5 to 20. Their lifespan averaged 8.67 days (N = 12, SD = 1.37).

Discussion

Resting eggs of *A. multiceps* have been misrepresented in the literature as spherical objects covered with short filaments (Voigt, 1957; Ruttner-Kolisko, 1974; Koste, 1978). The fuzzy eggs shown in these publications are subitaneous eggs that develop without arrest into females or males.

The error is traceable to Leydig (1854) and Weber (1898). Voigt (1957) credits Leydig (1854) for the figure of the, in his opinion, diapausing egg of *A. multiceps* presented in plate 84. The same figure is reproduced in Ruttner-Kolisko (1974) and Koste (1978). Leydig (1854) describes *A. multiceps* under its synonymous name: *Notommata myrmeleo* Ehrenberg, 1834. He assumed that the fuzzy eggs that were laid very rapidly were diapausing. However, both his diagram and description (page 24) of the so-called "Wintereier" fit the subitaneous egg perfectly. Leydig did not follow up on the development of these eggs. The only dissenting opinion (Plate, 1885) was disregarded by later authors. Weber (1898) produced a diagram of the adult female *A. multiceps* in which he designated a structure posterior to the vitellarium as an embryo. He noted on page 384 that the description was based on a few poorly preserved specimens. Nonetheless his diagram was passed along with minor modifications by Hyman (1951), Voigt (1957), Ruttner-Kolisko (1974), Koste (1978) and reinforced the misconception that *A. multiceps* is ovoviviparous (Koste, 1987; Shiel & Koste, 1993). Furthermore, investigators may have assumed that *Asplanchnopus* is ovoviviparous like other members of the Asplanchnidae. The fact that three widely separated populations of *A. multiceps* that were recently examined in Berlin (Schröder, T., personal communication, 1993) Mexico (Nandini & Sarma, 2005) and the United States (present communication) are oviparous contradicts the literature.

Although this report focuses on a single species, the faulty attribution of reproductive modes and structures may be more widespread due to the practice of passing along diagrams that were created decades or, in some cases over 100 years, previously with minor modifications from one publication to another. Species, like *A. multiceps*, that are seldom encountered are particularly susceptible to misrepresentation. Very dark diapausing eggs stuck to the substratum or vegetation may be missed because they blend in with the sediments. It is important to identify resting eggs correctly because their morphology is one of the criteria utilized in distinguishing closely related species (Gilbert & Wurdak, 1978). Resting egg morphology within a species is not invariable, however. In *A. multiceps* some eggs are dark all the way to the rim, while in others the dark interior is surrounded by a halo consisting of a clear zone followed by a relatively thin outer coat. These configurations represent different stages of development.

Securing the eggs to stable supports prevents them from being swept out into the open water and may also facilitate the emergence of the hatchling from the shell. Freshly-laid subitaneous eggs are not buoyant despite the presence of peripheral vacuoles and external filaments. If the egg is detached from the bottom of the Petri dish, lifted and released it will sink back down. The same holds for resting eggs. The fuzzy external coat may hinder the attachment of protozoa and fungi to subitaneous eggs although infected eggs are occasionally encountered. The vacuoles in the peripheral cytoplasm may be associated with the formation of the shell and/or the extrusion of the filaments that cover the surface. Subitaneous and resting eggs coated with short filaments are found in other rotifer genera, eg. *Epiphanes*, *Gastropus*, *Synchaeta* (Ruttner-Kolisko, 1974).

Daily tracking of the eggs laid by individual females in isolation failed to demonstrate the existence of amphoteric females in *A. multiceps*. Amphoteric females, capable of both mictic and amictic reproduction, have been identified in other species by Ruttner-Kolisko (1946 and 1977), Sudzuki (1955) and Gilbert (1995).

In their population growth study Nandini and Sarma (2005) demonstrated that fecundity and longevity in *A. multiceps* depend on diet. *A. multiceps* reared on *Plationus*, *patulus,* formerly *Brachionus*, *patulus*, had an average lifespan of 6 days and a highest net reproductive rate of 11 offspring. It is not clear, however, whether male offspring were included in their calculations.

Data collected from individual amictic and mictic females in this investigation vary somewhat from these values; the mean lifespan of both types of females is longer and their fecundity is lower. The discrepancy may be due to the fact that, in the current study, lifespan was measured from the egg stage onward, the temperature was 21^0C, versus 22 – 25^0C in Nandini and Sarma's (2005), and the diet was not optimized. The data show that the lifespans of amictic and mictic females do not differ significantly ($P = 0.642$), although their fecundity does ($P = 0.001$).

A. multiceps adult females are hardy; their eggs are large and easily collected with the aid of an Irwin loop. Subitaneous eggs develop on a predictable timetable and may be followed at the light and electron microscope level. The internal structure and development of diapausing eggs in rotifers has not been examined fully. Major questions remain regarding tissue organization in the dormant embryo. Wurdak et al. (1978) agree with Mràzek's (1897) observation that both the ectoderm and inner mass are syncytial. In a fully developed individual, however, some organs, eg. the stomach, consist of separate cells. The process of cell membrane restoration has not been studied. Under appropriate dietary conditions, the percentage of mictic females in *A. multiceps* can reach 80% or more. Consequently, a large number of diapausing eggs is available to investigate the resumption of development. Due to their size and firm consistency these eggs are easily handled. Developmental stages of subitaneous and resting-egg embryos can be compared in the same species as a result. Resting eggs currently in cold storage will be tested to uncover the stimulus required to initiate their further development. Other areas worthy of investigation include the adhesive properties of the eggs and the chemical identity of stored reserves in resting eggs.

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Figure Captions

Fig. 1 Amictic female *Asplanchnopus multiceps*

She is carrying a developing ovocyte, O, posterior to the stomach, S. The vitellarium, V, is visible beside the stomach.

Fig. 2 Male *A. multiceps*

The testis, T, contains typical and atypical spermatozoa.

Fig. 3 Mictic *A.multiceps* female

The developing ovocyte, O, is very dark and irregular in shape. The foot, F, on the right is one of the distinguishing features of the genus.

Fig. 4 Freshly-laid subitaneous egg

Note the hair-like projections that coat the shell and the vacuoles present in the peripheral cytoplasm. Based on its size this egg will develop into a female.

Fig. 5 Developing male embryo in the 4-cell stage

The large cell on the left is the precursor of the gonad and associated structures.

Fig. 6 Empty shell left behind by male hatchling

During hatching the shell splits into two symmetrical halves held together at the hinge.

Fig. 7 *A. multiceps* resting egg

The egg is perfectly round; the very dark interior is surrounded by a clear zone that is bounded by a thin external coat covered by short fibrils. The peripheral halo-like layer is off center.

Fig. 8 Scanning electron micrograph (SEM) of subitaneous egg

The fuzzy external coat is very pronounced.

Fig. 9 Surface detail of subitaneous egg

The adhesive patch, P, that secures the egg to the substratum covers a circular area on the underside.

Fig.10 High magnification of subitaneous egg surface

Note the filaments that extend from the shell. At their point of attachment to the shell they splay into rootlets. The surface is smooth in the area between the filaments.

Fig.11 SEM of *A. multiceps* resting egg

The surface is devoid of the filaments that characterize subitaneous eggs, but the adhesive patch, P, is present here as well.

Fig.12 Detail of resting egg surface

The piece of adhesive patch, P, on the right appears smooth while the general surface is marked by minute projections and tiny pores.


Fig 1
S
V
O
200 µm


Fig 2
T
200 µm


Fig 3
S
O
F
200 µm

Fig 4

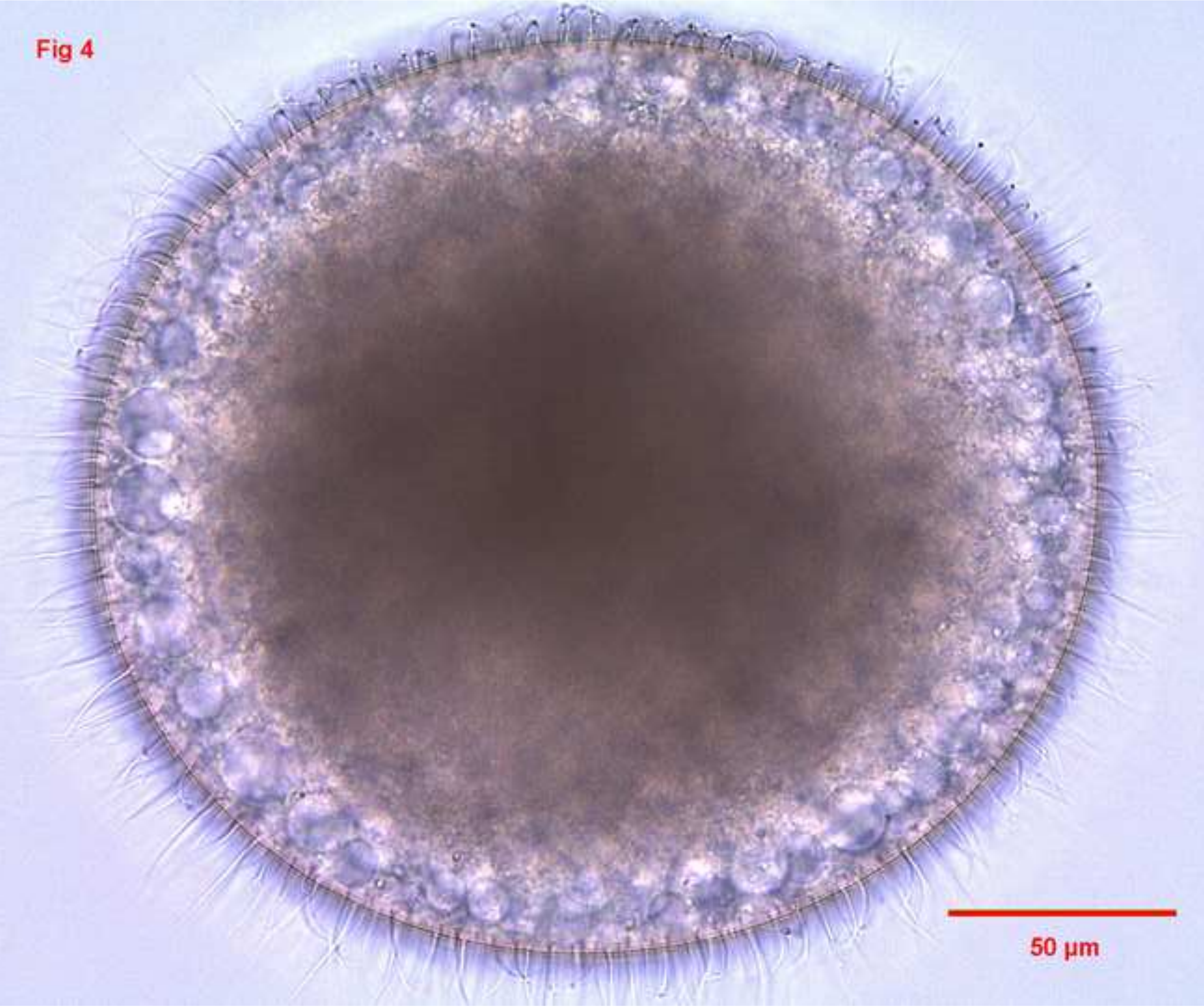

Fig 5


Fig 6
50 µm


Fig. 7
20 µm

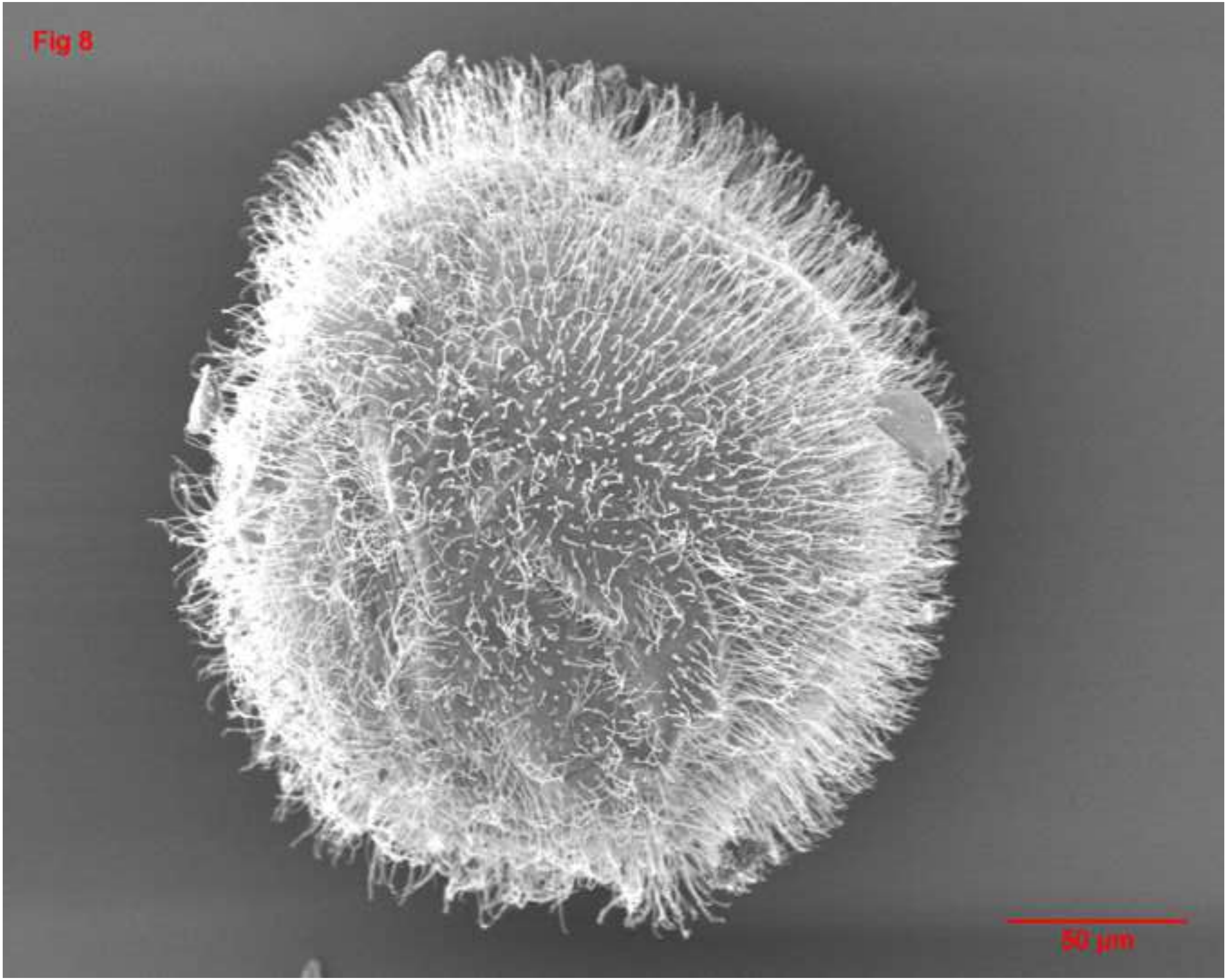
Fig 8
50 µm

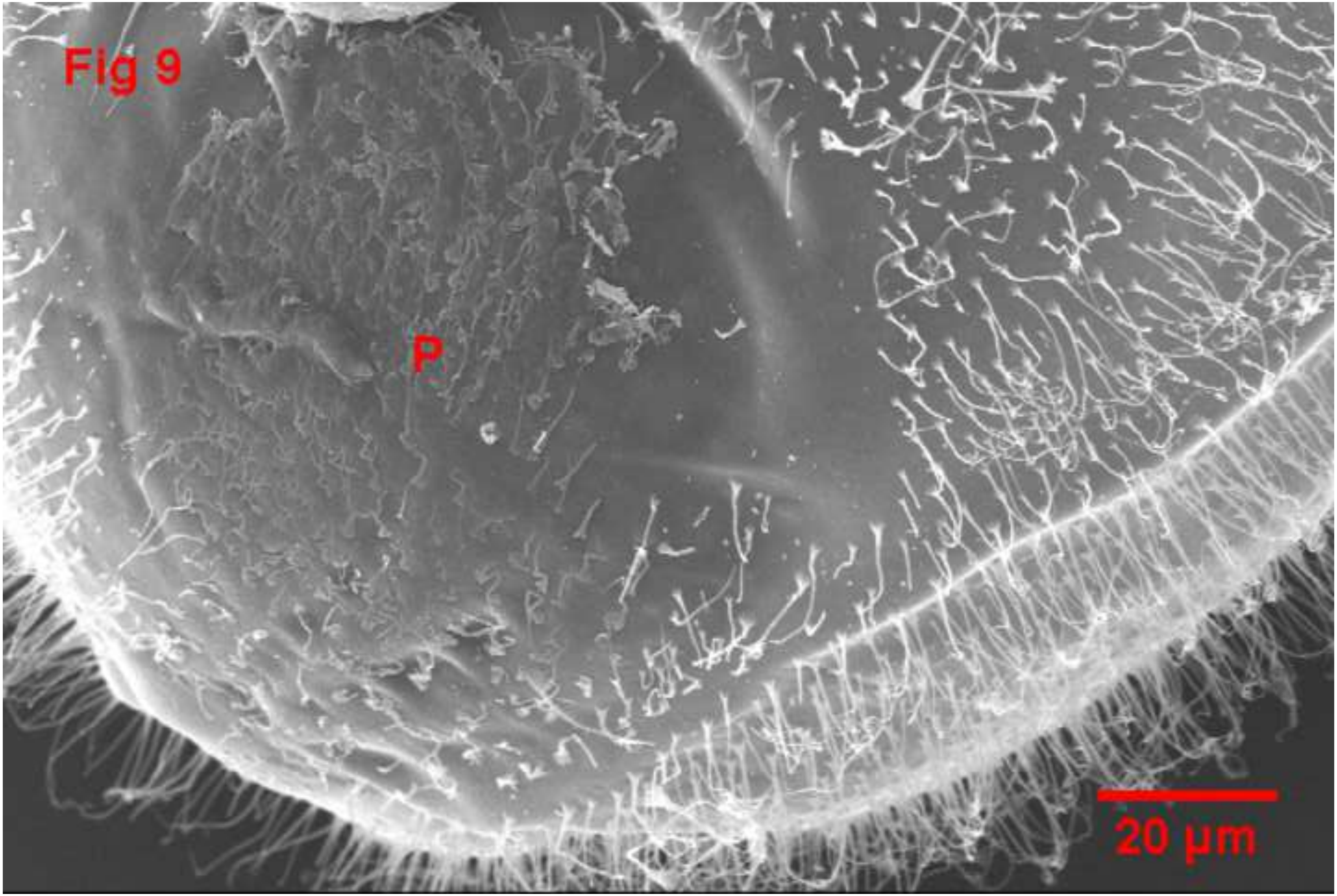
Fig 9
P
20 µm

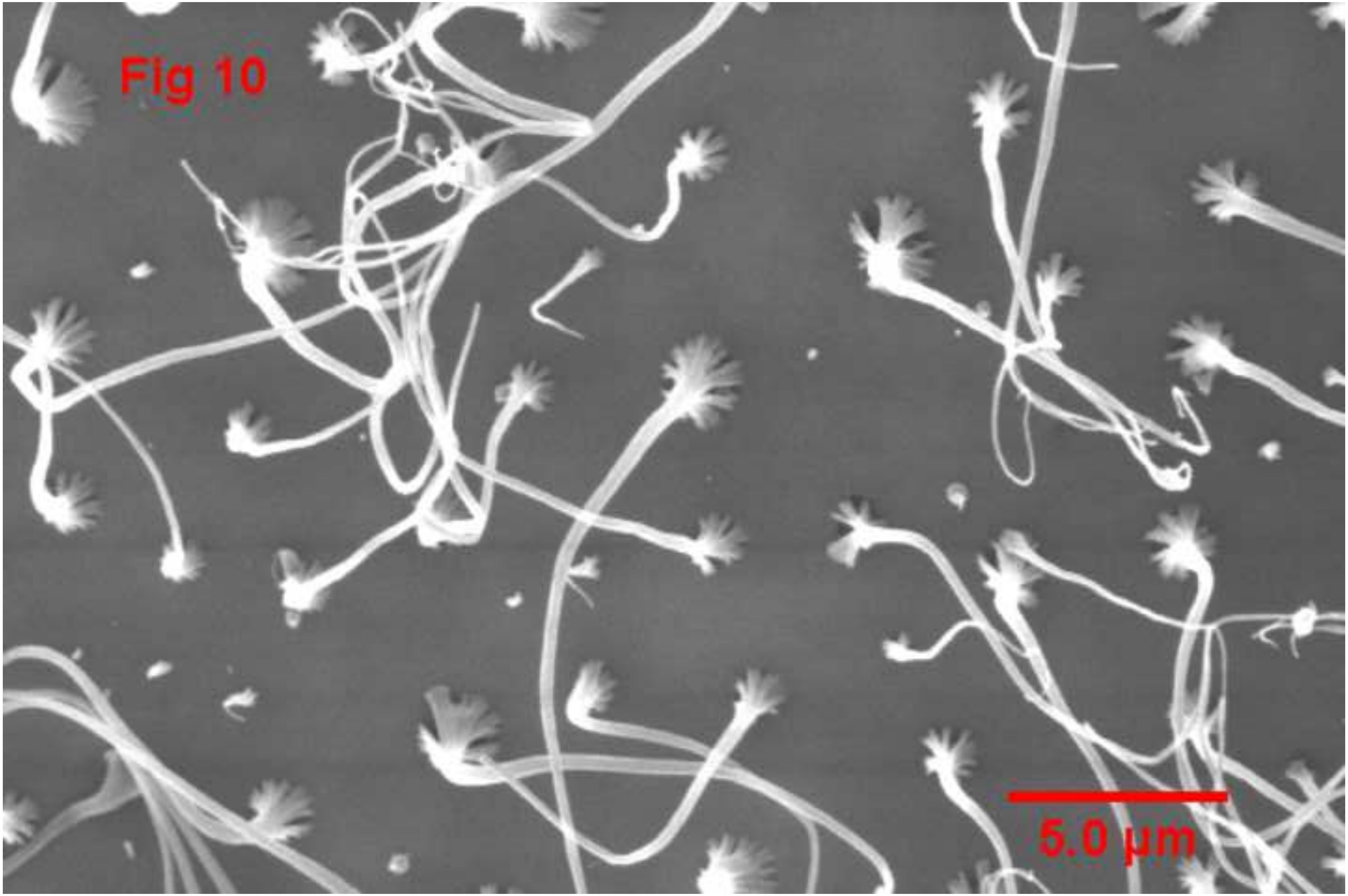
Fig 10
5.0 µm

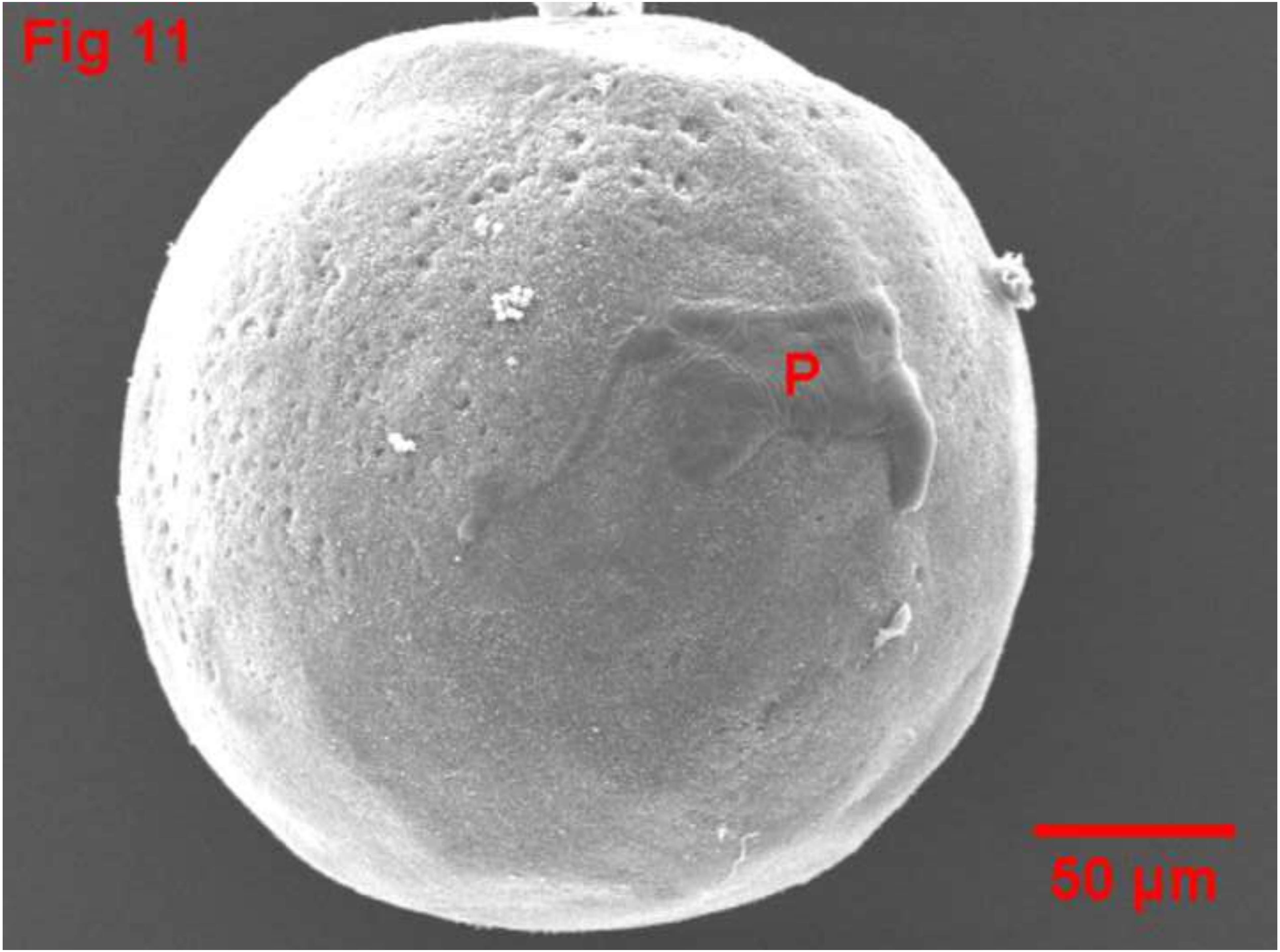
Fig 11
P
50 µm

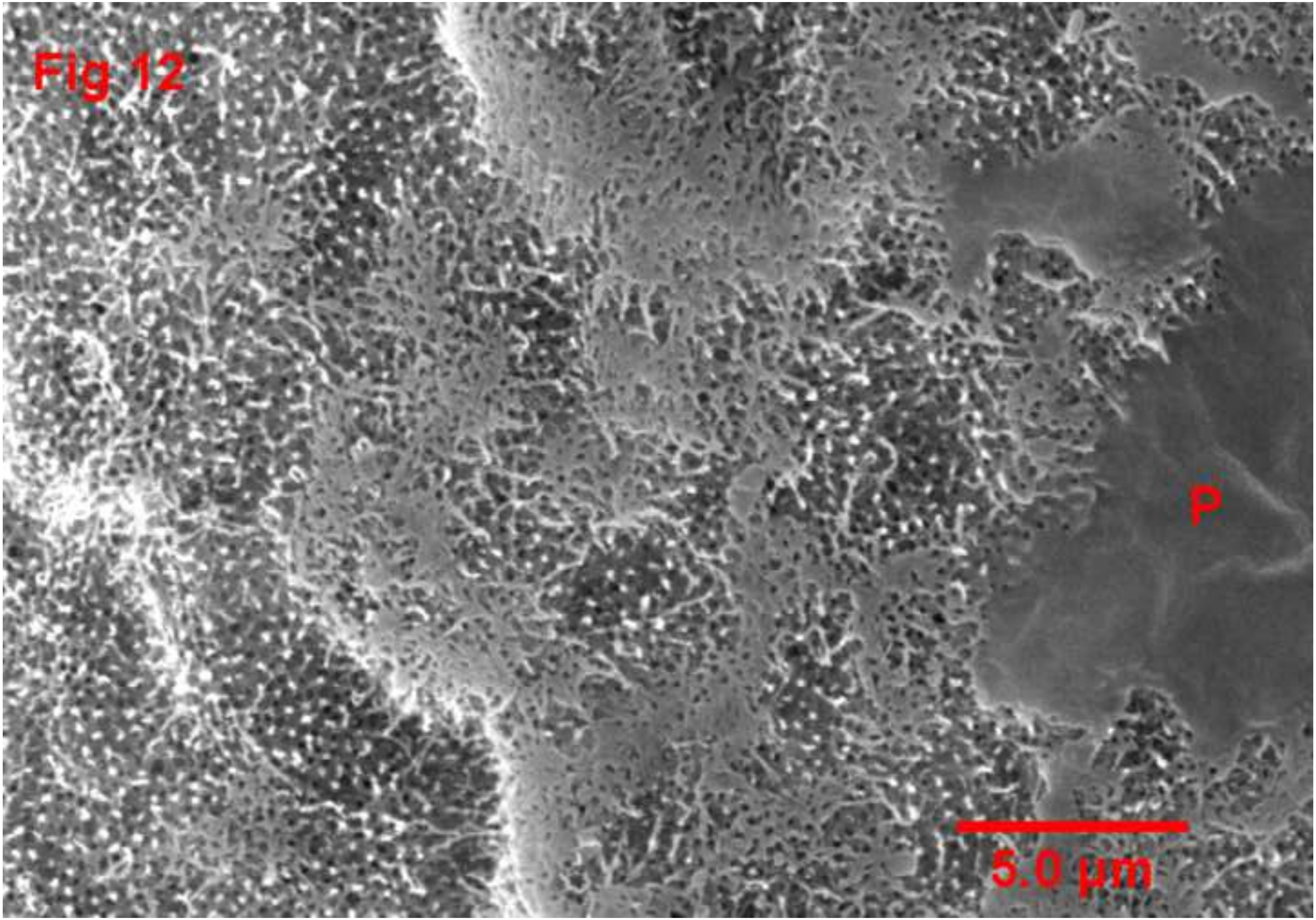
Fig 12
P
5.0 µm