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Can the protozoan parasite *Bonamia ostreae* infect larvae of flat oysters *Ostrea edulis* ?

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Abstract:

Bonamia ostreae is an intracellular protistan parasite affecting flat oysters *Ostrea edulis*. It can be detected in juveniles but mortalities mainly affect oysters which are more than 2 years old. The parasite is usually observed inside haemocytes and sometimes free, notably in gill epithelia suggesting a parasite release through this organ. However, the infective form and ways of entry and release remain undetermined. Flat oysters incubate their larvae in their pallial cavity for 8–10 days before releasing them into the water column. Flat oysters in Bay of Quiberon in South Brittany (France) are known to be infected with *B. ostreae* since 1979 and is the most important area in France for *O. edulis* spat collection. Flat oysters incubating larvae were sampled in this area during summertime between 2007 and 2009. Both adults and larvae were preserved and assayed by PCR and *in situ* hybridisation (ISH). PCR tests revealed the presence of parasite DNA in some adults and larvae. Specific labelling could be detected by ISH in gills, digestive system, gonad and mantle in adults and in the epithelium surrounding the visceral cavity of some larvae. Our results demonstrate that larvae can be infected with *B. ostreae*. Larvae might thus contribute to the spread of the parasite during their planktonic life. In addition, their transfer for aquaculture purpose should be controlled especially when they are exported from infected zones.

Keywords: *Bonamia ostreae*; Flat oyster; *Ostrea edulis*; Transmission; Larvae; Parasite life cycle

1. Introduction

The flat oyster *Ostrea edulis* is a native species from Europe. It occurs along the western European coast from Norway to Morocco, through the Mediterranean Sea, and into the Black Sea. Naturalized populations also occur along the eastern coast of North America from Maine to Rhode Island following intentional introductions in the 1940s and 1950s (Hidu & Lavoie, 1991; MacKenzie et al. 1997). This species has been endangered by overfishing, cold winters, predation pressure and diseases and is today in the OSPAR (Oslo and Paris Conventions for the protection of the marine environment of the North-East Atlantic) list of threatened and/or declining species and habitats (OSPAR agreement 2008-6). Specifically, French flat oyster production estimated at 28 000 tonnes in 1960 dramatically decreased because of two protozoan diseases namely marteiliosis due to *Marteilia refringens* and bonamiosis due to *Bonamia ostreae* (Meuriot & Grizel, 1984; Goulletquer & Héral, 1997). The production of this endemic species has remained low, less than 2 000 tonnes per year since the emergence of these two diseases and is now located in a few specific areas (Figure 1). Data from the last census on shellfish culture in France carried out in 2001 showed that most of the spat is collected in the Bay of Quiberon and to a lesser extent in the Bay of Brest (Girard et al. 2005). Spat collected in the Bay of Brest and some of the spat collected in the Bay of Quiberon are moved to Cancale, North Brittany for grow-out when the spat is 10 months old. In 2001, the production of flat oysters was estimated at 1 960 tonnes (FAO, 2008).

Few data are available on pathogens affecting young flat oysters. However, herpes-like viruses were reported in 5-month-old spats collected in northern Brittany (Comps & Cochennec, 1993). Similar viruses were associated with the mortality of flat oyster larvae in a hatchery (Renault et al. 2000, Arzul et al. 2001). *Vibrio* strains were shown to be pathogenic

to larvae of flat oysters by inducing mortalities in hatcheries (Jeffries, 1982; DiSalvo et al. 1978; Tubiash et al. 1965). Young prespawning flat oysters (1-3 month old to 18 month-old) are susceptible to infection by *B. ostreae* and can develop a high prevalence and intensity of infection over a six-month period (Lynch et al. 2005). Mortality associated with infection with *B. ostreae* has even been described in 6 month-old juveniles (Lallias et al. 2008). However, individuals older than 2 years appear more susceptible to the disease (Balouet et al. 1983; Cullotty & Mulcahy, 1996; Grizel, 1985; Robert et al. 1991) and death usually occurs concurrently with the highest level of infection intensity (Bréhelin et al. 1982; Caceres-Martinez et al. 1995; Montes et al. 2003). While adults and juveniles are known to be susceptible stages to bonamiosis there is no data on the possible role of larvae in the cycle of the parasite.

Bonamia ostreae life cycle is unknown, but the disease can be transmitted directly between oysters in a population or experimentally by cohabitation or inoculation (Elston et al. 1986, Hervio et al. 1995), suggesting that an intermediate host is not required for the parasite to complete its life cycle. Observation of free parasites in gill epithelia potentially associated with gill lesions supports the hypothesis of a parasite release through this organ (Montes et al. 1994). However, the infective form and routes of entry and release remain undetermined. A controversial description proposed that *B. ostreae* was an ovarian tissue parasite for part of its life cycle (Van Banning, 1990) but this hypothesis was not confirmed. In spite of several management practices, diseases have drastically affected wild and cultured flat oyster populations. The main solutions for the industry rely on transfer restrictions and on the development of resistant strains which require a better understanding of host pathogen interactions.

The Bay of Quiberon, South Brittany, France (Figure 1) is an interesting site to study bonamiosis in flat oyster populations because flat oysters there have been infected since 1979

with prevalence of infection ranging from 2 to 37% and a mean around 13% (Arzul et al. 2006). In addition, it is the most important bay for flat oyster spat collection in France and surveys on the reproduction of this species have been carried out there since 1996. Flat oyster female gametes are liberated into the pallial cavity where they are fertilized by externally released sperm. After an incubation period of 8-10 days, larvae (160 µm in size) spend 8 to 10 days as a pelagic stage before settlement. The survey of flat oyster reproduction in the Bay of Quiberon aims at following the status of spawners to determine the presence of gametes and larvae in the oysters and the presence of larvae in the water column in order to advise farmers about the most suitable period for spat collection. In the context of this survey, some adults incubating larvae in their pallial cavity were selected in 2007, 2008 and 2009, and were tested for the presence of the parasite *B. ostreae.* The detection of the parasite in adults and juveniles raises the question about the transmission of the parasite from spawners to larvae as well as the role played by larvae in the disease spread.

2. Materials and methods

2.1. Study site

The Bay of Quiberon is an open bay located in southern Brittany (Figure 1). This bay has an area of 2873 ha and presents suitable conditions for oyster production: depth between 3 and 10 m allowing mitigation of swell effect and dredge use for harvesting; good water mass renewal (SHOM 1968); fertilization by close rivers (Loire and Vilaine); protection by Quiberon peninsula and good sediment composition (sand and mud). Eighty three oyster farms are located in this bay (Mazurié et al. 2002). Most of these farms produce the Pacific cupped oyster *Crassostrea gigas*. However, shallow sites in the North and West of the Bay are dedicated to flat oyster spat collection on mussel shells or on limed plates. Twenty two

oyster farms collect the spat of *Ostrea edulis* and about 12 of them grow-out flat oyster for marketing.

2.2. Flat oyster sampling

Spawners were collected weekly by diving between the end of April and the end of August. Spawners were then opened and oysters incubating larvae (Figure 2) were selected for our study. Thirty one, 53 and 36 oysters were found incubating larvae in 2007, 2008 and 2009 respectively.

A section of tissue from each adult oyster was fixed in Davidson's fixative for histology and *in situ* hybridisation tests and a piece from gills was also fixed in 100 % ethanol for DNA extraction. Incubated larvae from each adult oyster were transferred in separate tubes and some were fixed in 100 % ethanol for DNA extraction while the remainder were preserved in Davidson's fixative for *in situ* hybridisation tests. Prior to DNA extraction, larvae were rinsed once in 1X PBS (150 mM NaCl, 12.5 mM Na_2HPO_4, 3 mM KH_2HPO_4, pH 7.5).

2.3. DNA extraction

Twenty five mg of gill tissue or larvae were collected from each adult for DNA extraction using the QIAamp DNA minikit (Qiagen) according to the manufacturer's instructions. DNA was eluted and resuspended in a final volume of 50 µl of sterile deionised water and then diluted at a final concentration of 100 ng/µl.

2.4 PCR conditions and parasite species determination by RFLP and sequencing

Conventional PCR was performed according to Cochennec et al. (2000). PCR reactions were carried out in a final volume of 50 µl. Between 50 and 100 ng of extracted DNA were added to 49 µl of PCR mixture containing buffer (500 mM KCl, 100 mM Tris/HCl [pH 9.0 at 25°C]

156 and 1% Triton® X-100), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 µM forward (Bo) and reverse
157 (Boas) primers and 0.02 units/µl Taq DNA polymerase (Goldstar Eurogentec). Amplification
158 programme was as follows : initial denaturation for 5 min at 94°C; 30 cycles of 1 min at
159 94°C, 1 min at 55°C, 1 min at 72°C and a final extension of 10 min at 72°C.

160 Parasite species was subsequently determined by restriction fragment length polymorphism
161 (RFLP) analysis (Cochennec et al. 2003; Hine et al. 2001) performed by separate digestions
162 of 10 µl of PCR products with *Bgl*I and *Hae*II (Promega). The resulting fragment patterns
163 were analysed electrophoretically on 2% agarose gel.
164 Some PCR products were cloned using the TOPO TA cloning kit (Invitrogen) according to
165 manufacturer's recommendations and positive clones were then selected for plasmid DNA
166 purification by FastPlasmid® Min (Ependorf). Some plasmidic DNA suspensions were
167 sequenced bidirectionally using the Big Dye V3 sequencing kit (Applied Biosystem) and Bo
168 and Boas primers. Obtained sequences were compared with those included in GenBank using
169 BLAST algorithm (Atschul et al. 1997).

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172 *2.5. Histology and in situ hybridisation*

173 After 48 hours of fixation in Davidson's fixative, tissue were maintained in 70 % ethanol until
174 dehydration and embedding in paraffin for histology according to standard procedures. Larval
175 samples were treated similarly to adults but in small plastic tubes. Paraffin blocks were cut in
176 2-3 µm sections and stained by hematoxylin and eosin. *In situ* hybridisation was performed on
177 5 µm thick sections on aminoalkylsilane coated slides (Silane-Prep Slides, Sigma). The probe
178 was labelled by means of digoxigenin incorporation to the PCR reaction mix. PCR was
179 performed as described above, except that 2.5 µl of DIG-dUTP 25 mM (Roche) were added to
180 the mix. *In situ* hybridisation was performed following procedures previously published

(Cochennec et al. 2000) and using non infected oysters *Ostrea edulis* as negative controls and *Ostrea edulis* infected with *B. ostreae* as positive controls.

3. Results

3.1. Detection by PCR and species characterization of Bonamia sp.

In 2007, four of the 31 tested adults yielded positive signal by PCR. One of the 31 related larvae samples appeared positive by PCR and it corresponded to one of the positive adult oyster. In 2008, of the 53 tested samples, 13 adults and 9 samples of larvae were found positive by PCR. Three of the spawners detected positive had positive signal in their corresponding larvae samples. In 2009, 8 of 36 adults and 5 of 36 samples of larvae were detected positive by PCR. All 5 positive larval samples corresponded to positive adult oysters. PCR products were tested by RFLP and all the obtained restriction profiles were identical to *B. ostreae* except for one sample of larvae collected in 2008 which exhibited *Bonamia exitiosa* like profiles (Figure 3).

Six PCR products showing *B. ostreae* RFLP profiles and the one showing the *B. exitiosa* profile were cloned. A total of 40 clones were tested again by PCR-RFLP to check the potential presence of both parasite species in the same sample. Larval samples which gave *B. exitiosa* profiles by direct PCR-RFLP analysis gave only *B. exitiosa* profiles for the 4 tested clones. The other PCR products only yielded *B. ostreae* profiles.

One clone (a) showing *B. ostreae* profile and two clones (b and c) showing *B. exitiosa* profiles were selected for sequencing. Sequencing confirmed the RFLP results: obtained sequences showed (a) 3 transitions and 99% of identity compared with *B. ostreae* (AF192759.1) and (b and c) 100% of identity with *B. exitiosa* (F337563.1).

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209 *3.2. Histology*

210 In adults, histology revealed the presence of *Bonamia* parasites in 2 (6%), 9 (17%) and 4
211 (11%) of the examined flat oysters in 2007, 2008 and 2009 respectively (Table 2). Seventy
212 three percent of these 15 oysters were also found positive by PCR. By histology, infection
213 level was generally low (few infected zones and few parasite cells observed per infected area)
214 and the parasite was mainly detected in haemocytes in the connective tissue of the digestive
215 system, gonad and gills (Figures 4a, b, c and d). Infection with the parasite was generally
216 associated with haemocyte infiltration. However, haemocyte infiltration was observed in
217 many tested oysters, from 55% in 2008 up to 65% in 2007.
218 Histological examination also revealed the presence of other microorganisms commonly
219 observed in flat oysters including *Mytilicola,* ciliates, turbellaria and rickettsia-like organisms
220 (Table 2). Haemocytic neoplasia and abnormal nuclear shapes were observed in 1 and 3
221 oysters collected in 2009, respectively (Table 2).

222

223 *3.3. In situ hybridisation (ISH)*

224 ISH tests were performed on adult and larval samples found positive for *B. ostreae* by PCR.
225 In 2007, only adult oysters could be tested by ISH since larvae were not fixed for that
226 purpose.
227 Thirty one adults detected positive by PCR and/or histology were tested by ISH and positive
228 signal was observed in 13 of them. The parasite was mainly detected in the gills (in 8 out of
229 the 13 positive oysters), in the gonad (in 7 out of the 13 positive oysters), in the digestive
230 system (in 6 out of the 13 positive oysters) and in the mantle (in 2 out of the 13 positive
231 oysters). In gills and digestive system, the parasite was detected in haemocytes in the
232 connective tissue (Figure 5a) and extracellularly in the epithelium. In the gonad, the parasite

was mainly detected in haemocytes inside the lumen of gonadal follicles (Figure 5b ; Table 3). In three adults, some larvae present in the sections appeared positive.

Fourteen pools of larvae detected positive in PCR and histology were tested by ISH and 7 gave positive signals. The parasite was essentially observed in the epithelium surrounding the visceral cavity (Figures 6a, b and c).

4. Discussion

Bonamia ostreae is an intracellular protozoan affecting flat oysters *Ostrea edulis*. Despite more than 30 years of research, its complete life cycle remains unsolved and several characteristics of the disease are not understood. In particular, the routes of entry and release of the parasite from its host are undetermined. Parasite release could take place in gills as supported by observation of free parasites in this organ (Montes et al. 1994). Moreover, Van Banning (1990) suggested that *B. ostreae* was an ovarian tissue parasite for part of its life cycle but this hypothesis has not been confirmed. While adults and juveniles are known to be susceptible stages to bonamiosis, the possible role of larvae in the cycle of the parasite was unknown.

We took advantage of a survey on flat oyster reproduction in the Bay of Quiberon, the most important French area for spat collection of flat oyster, which is also known to be endemic for *B. ostreae* since 1979. In the frame of this survey, oysters incubating larvae were sampled for subsequent analyses to test the presence of the parasite in spawners and in their progeny.

During the three years of the study, adults (between 13 and 25%) were found positive by PCR more frequently than larval samples (between 3 and 17%). Thirty six percent of adults found positive by PCR had their corresponding pool of larvae positive. All the PCR positive samples appeared infected with *B. ostreae* except one larval sample found infected with *B.*

exitiosa. *Bonamia exitiosa* and *B. ostreae* are very similar morphologically and their differential diagnostic is very difficult based on histological examination. PCR-RFLP and sequencing are necessary to characterize these parasites at the species level. In October 2007, *B. exitiosa* was reported for the first time in flat oyster *Ostrea edulis* in Europe, more precisely in Galicia, Spain (Abollo et al. 2008). It was then reported in Italy along the Adriatic coast (Narcisi et al. 2010) and in France in the Mediterranean Sea. The positive result obtained in one larvae sample in the Bay of Quiberon in 2008 is the only detection of *B. exitiosa* DNA on the French Atlantic coast. More samples should be tested to determine the prevalence of this parasite in Quiberon bay.

As expected, histology appeared less sensitive than PCR but depicted the distribution of the parasite inside the oyster and revealed the presence of other pathogens and associated lesions. Infection level was low and *B. ostreae* was mainly detected in haemocytes in the digestive system, gonad and gills. Haemocyte infiltration was observed in many tested oysters and not only in *Bonamia* infected oysters. Adult oysters and larvae found positive by PCR were also tested by *in situ* hybridisation. In adult oysters, the parasite was detected, in descending detection frequency, in the gills, gonad, digestive system and in the mantle. It was observed inside haemocytes in connective tissues and in gonadal follicles or extracellularly in the epithelia of the gills and the digestive system.

Flat oysters tested in our study were male (53%) or hermaphrodite (47%). The larviparous oysters of the genus *Ostrea* including *O. edulis* undergo rhythmical changes in sexuality, the initial phase in these species is usually male, followed by alternating female and male phases (Orton, 1927). This feature explains why in our study, incubating oysters were male or hermaphrodite. The adult broods their larvae inside the pallial cavity. In the congeneric species *O. chilensis,* the embryos are also brooded in the pallial cavity of the parent oyster, within which they move constantly and freely while maintaining a close association with the

286 maternal demibranchs, passing along the food grooves and aggregating in the region of the
287 labial palps (Chaparro et al., 1993). In our study, seven pools of larvae presented positive
288 signal by *in situ* hybridisation. In addition, three larvae present in sections of infected adults
289 were found infected using this technique. In positive larvae, the parasite is located in the
290 epithelia surrounding the visceral cavity. This location suggests that larvae become infected
291 through the ingestion of parasites. In *O. chilensis*, it has been shown that larvae are able to
292 remove suspended particles, between 2 and 10 µm in diameter, from the pallial cavity of the
293 brooding adult and ingest them (Chaparro et al. 1993).
294 The results of *in situ* hybridisation demonstrated that larvae of *O. edulis* can be infected with
295 *B. ostreae* and that PCR positive results do not only correspond to the presence of DNA from
296 dead parasites or from parasites attached to the surface of the larvae.
297 The detection of the parasite in adults and larvae raises, among others, the question about the
298 transmission of the parasite from adults to larvae. The flat oyster *O. edulis* keeps eggs and
299 then larvae for an incubation period of 8-10 days before releasing larvae into the water
300 column (Marteil, 1960). This period of incubation might favour the transmission of the
301 pathogens from the adult oyster to its progeny. A previous study carried out on *O. edulis*
302 families suggested a significant influence of the Ostreid herpesvirus 1 infective status of the
303 parents on the infection of the progeny and thus a possible vertical transmission of the virus .
304 (da Silva et al. 2008). In our study, the level of infection in adult oysters was low which could
305 explain the low level of infection observed in larvae by *in situ* hybridisation. Generally, few
306 larvae were found positive and few parasites were detected in infected larvae.
307 The success of experimental transmission of the parasite between infected and non infected
308 oysters demonstrates that direct transmission is possible through the water column and an
309 intermediate host is not required for the parasite to complete its life cycle (Hervio et al. 1995).
310 These results are supported by recent data showing that *B. ostreae* is able to survive in sea

311 water for at least 1 week (Arzul et al. 2009) and can thus be efficiently transmitted through
312 the water.
313 However, it seems that *B. ostreae* is able to use additional routes of transmission. Lynch et al.
314 (2007) investigated the potential involvement of macroinvertebrate and zooplankton species
315 in the parasite life cycle and found that eight benthic macroinvertebrates and 19 grouped
316 zooplankton samples gave positive results by PCR. Certain species, found positive for the
317 parasite DNA, were then used in laboratory transmission trials, to investigate if they could
318 infect naïve oysters. Transmission of *B. ostreae* was effected to two naïve oysters cohabiting
319 with the brittle star, *Ophiothrix fragilis* (Lynch et al. 2006). Nevertheless, considering the
320 correlation between density of oysters and prevalence of bonamiosis (Grizel 1985, Hudson &
321 Hill 1991), the parasite mainly depends on flat oysters *O. edulis* for its survival and spread,
322 and other aquatic organisms might not be involved as important carriers or transmitters (Van
323 Banning 1988). Therefore, transmission of *B. ostreae* between oysters probably mainly occurs
324 through the water column and larvae may contribute to facilitate the dispersal of the parasite
325 during their planktonic life and transport by the currents in the zone.
326
327 To conclude, positive results obtained by PCR and confirmed by *in situ* hybridisation are
328 indicative of an infection of adults but also of larvae by the parasite *B. ostreae*. For the first
329 time it is shown that *B. ostreae* is able to infect oyster larvae within the pallial cavity. These
330 results suggest that the parasite could be transmitted from adults to larvae during the period
331 of larval incubation. Larvae might thus contribute to spread the parasite during their
332 planktonic life. In addition, the transfer of all life stages of the oyster for aquaculture or
333 stock enhancement purpose should be controlled especially when they are exported from
334 infected zones.
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TABLES

Year	Positive Adults	Positive pools of larvae	Adult/Larvae	Total
2007	4	1	1	31
2008	13	9	3	53
2009	8	5	5	36

Table 1- Results of PCR for adults and pools of larvae tested in 2007, 2008 and 2009 for the presence of *Bonamia* parasite. The number of adult oysters for which the corresponding pools of larvae were found positive by PCR is indicated in the column "Adult/Larvae". The total number of tested samples is reported in the last column.

Year	2007 *N*=31	2008 *N*=53	2009 *N*=36
Bonamia spp.	2	9	4
Haemocyte infiltration	20	29	23
Mytilicola spp.	1	3	
Ciliates	4	1	2
Rickettsia-like organisms	1		1
Necrosis of digestive epithelia	1	5	3
Turbellaria	1		
Atrophy of digestive diverticula		1	
Abnormal nuclear shapes			3
Haemocytic neoplasia			1

Table 2- Observation by histology of pathological conditions in flat oysters *Ostrea edulis* incubating larvae.

Gills	8	Connective tissue (IH) Epithelium (EC)	6 5
Mantle	2	Connective tissue (IH) Epithelium (EC)	2 0
Gonad	7	Connective tissue (IH) Follicles (IH)	1 7
Digestive system	6	Connective tissue (IH) Epithelium (EC)	5 4

Table 3- Tissue distribution of the parasite detected by *in situ* hybridisation in positive oysters; Number of positive oysters according to the organ and the tissue (*N*= 13). IH: Intrahaemocytic; EC: Extracellular

FIGURE LEGENDS

Figure 1- Main French sites concerned by the production of flat oyster, *Ostrea edulis*: spat is mainly collected in Bay of Quiberon and the Roadstead of Brest; some of the oysters are then moved to Cancale for grow-out. Detail of Bay of Quiberon, Southern Brittany, France

Figure 2 – Flat oyster incubating larvae

Figure 3 – Restriction profiles obtained after digesting Bo-Boas PCR products with *Bgl*I. Samples of larvae (numbers 13, 19, 20, 21, 30, 36 and 43) displayed restriction profiles similar to *Bonamia ostreae* (T+ Bo ost) while sample number 28 was not digested by *Bgl*I like that of *B. exitiosa* (T+ Bo ex). First line corresponds to a 100 bp ladder (Smartladder, Eurogentec).

Figures 4a, b, c and d – Hematoxylin and eosine stained sections of adult flat oysters.

509 4a- Haemocyte infiltration in the connective tissue of gills.

510 4b- Detail of 4a showing some *Bonamia ostreae* cells (arrows) inside the cytoplasm of
511 haemocytes. One parasite is binucleated (arrow head).

512 4c- Presence of *Bonamia ostreae* (arrows) in some hameocytes in the lumen of a gonadal
513 follicle.

514 4d - Detail of 4c showing some *Bonamia ostreae* cells (arrows) inside the cytoplasm of
515 haemocytes.

516

517 Figures 5a and b- *In situ* hybridization assay on adult flat oysters

518 5a- Positive signal is observed in the connective tissue of gills

519 5b- Positive signal is observed in the lumen of a gonadal follicle

520

521 Figures 6a, b and c- *In situ* hybridization assay on larvae of flat oysters- Positive signal is
522 osberved in cells surrounding the visceral cavity.

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Figure

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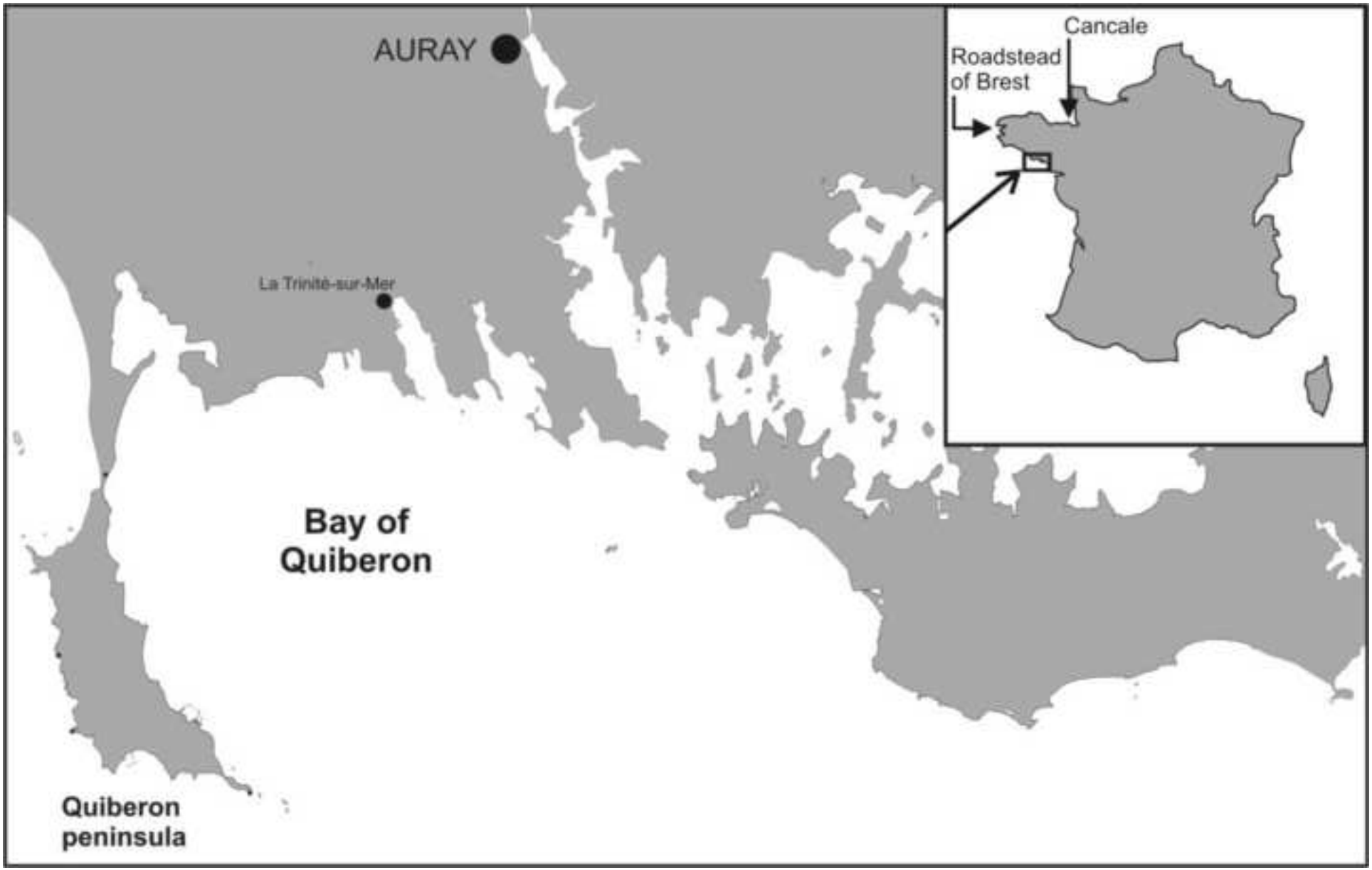

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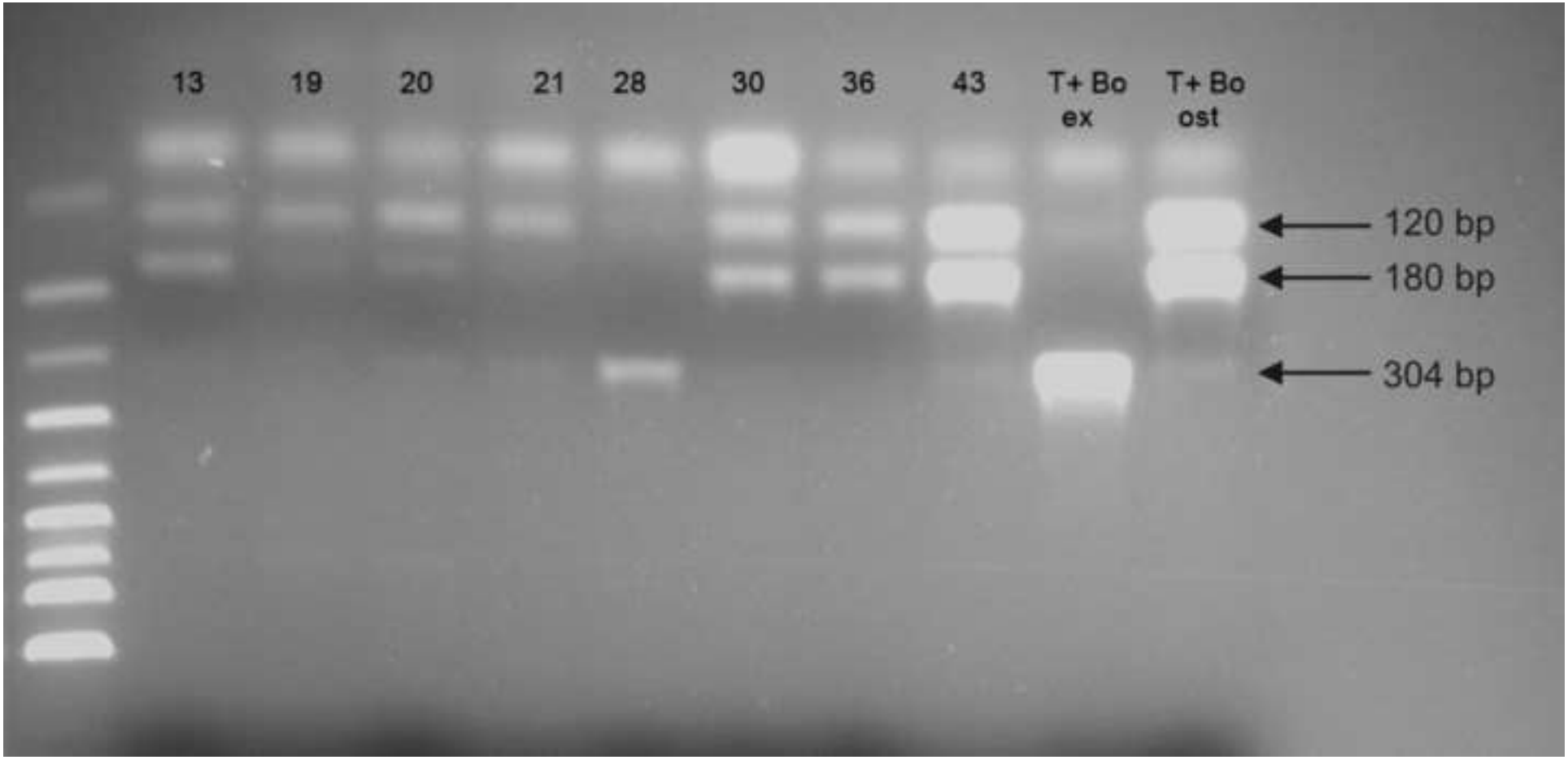

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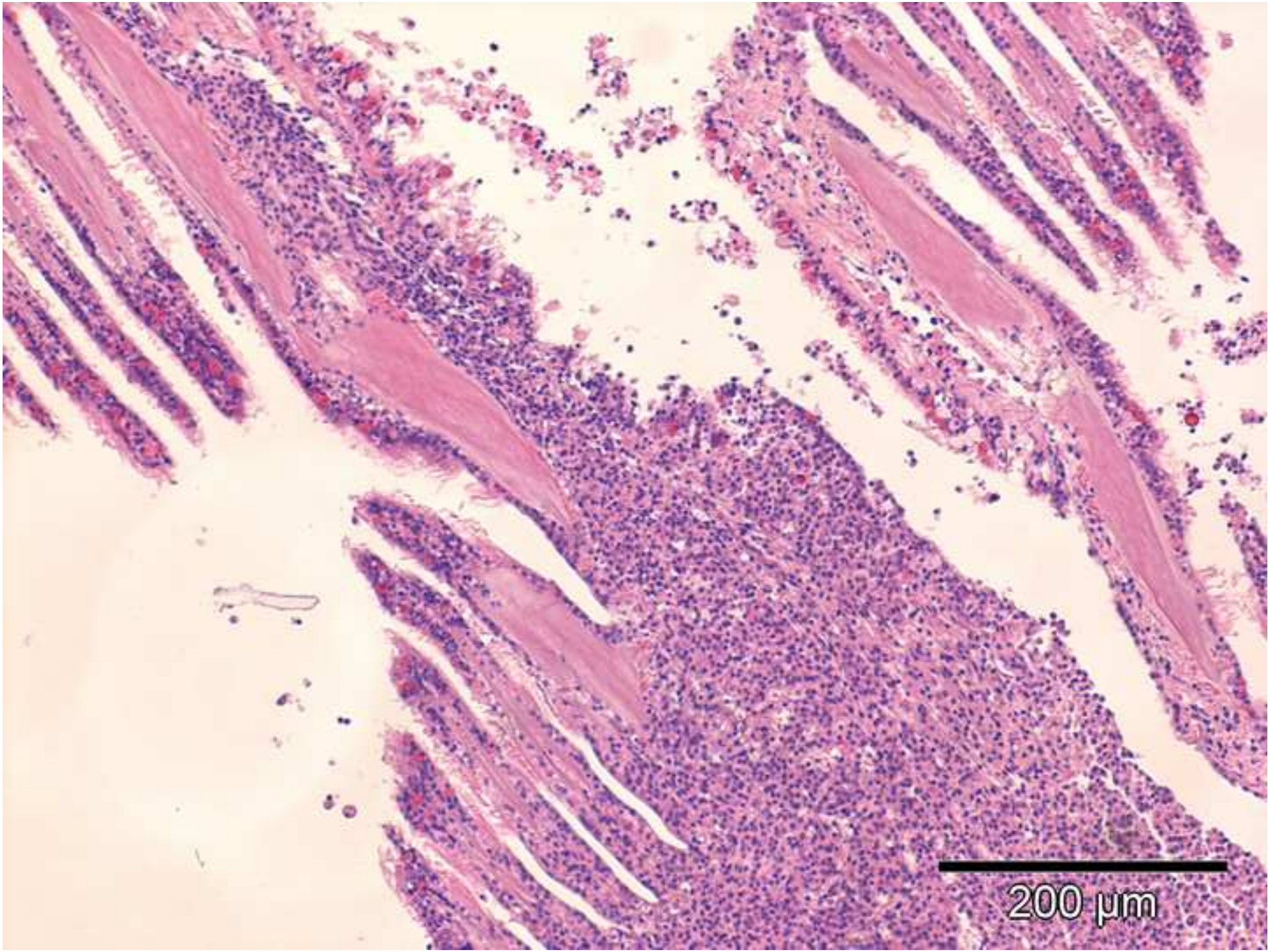

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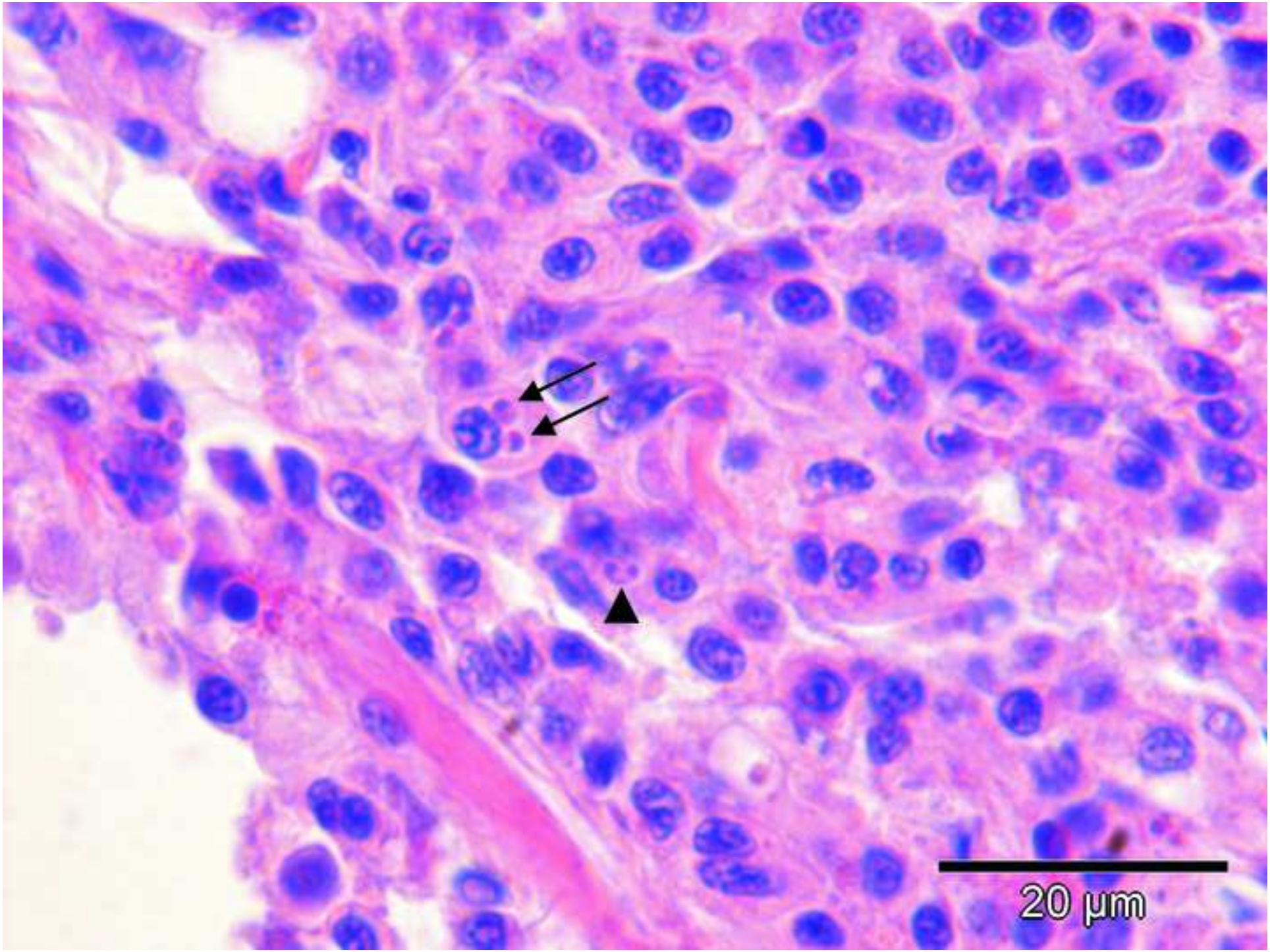

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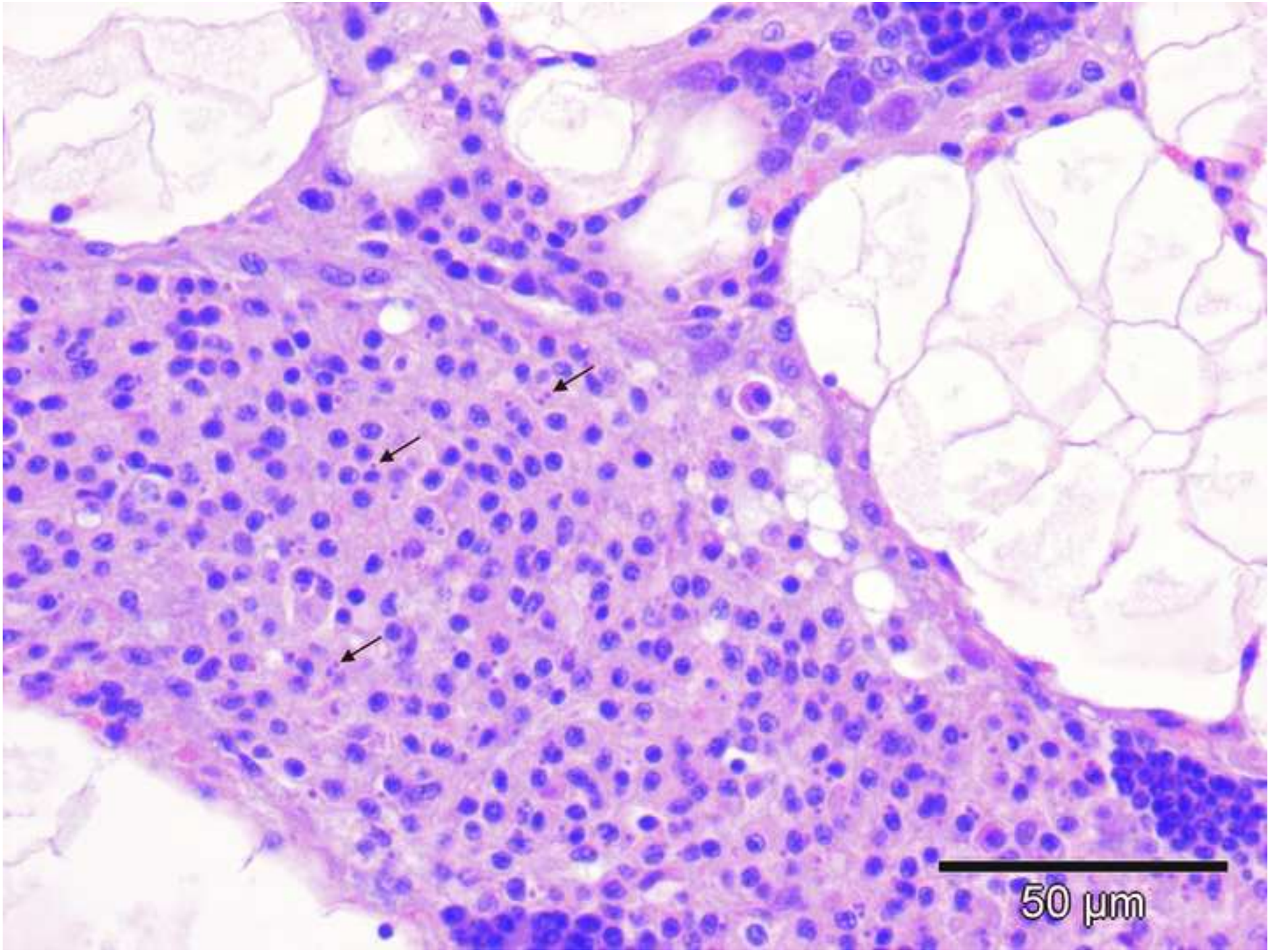

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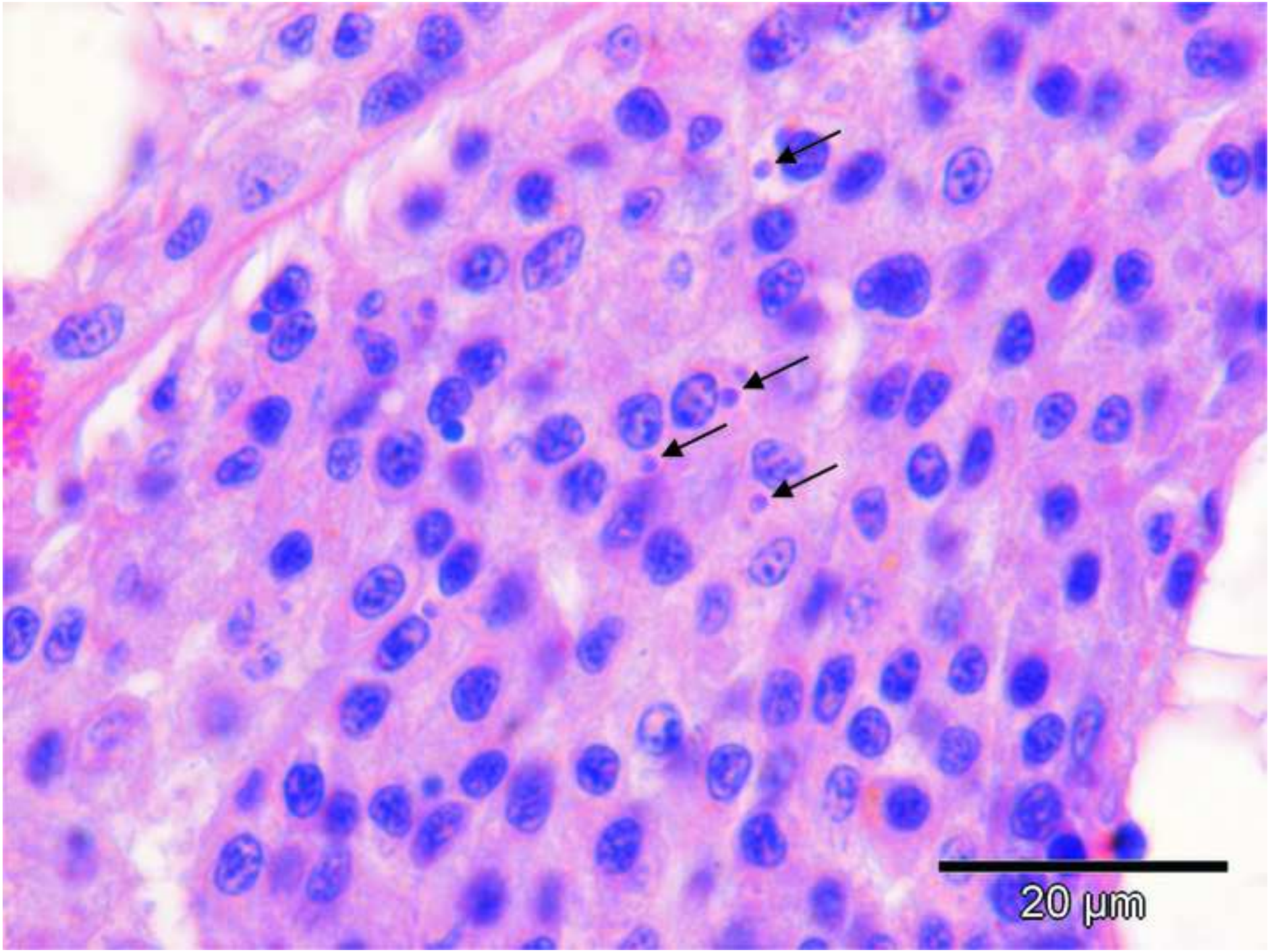

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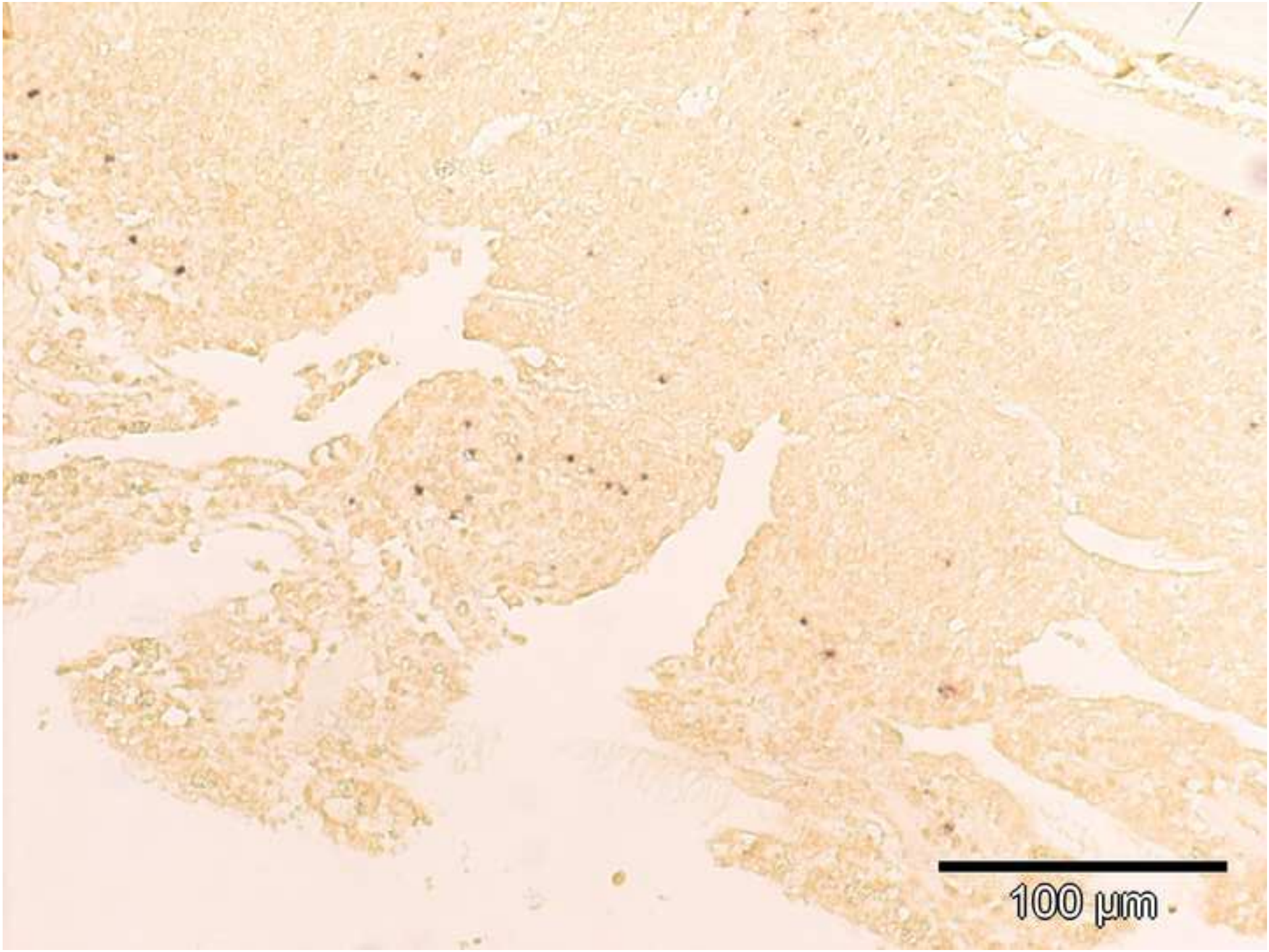

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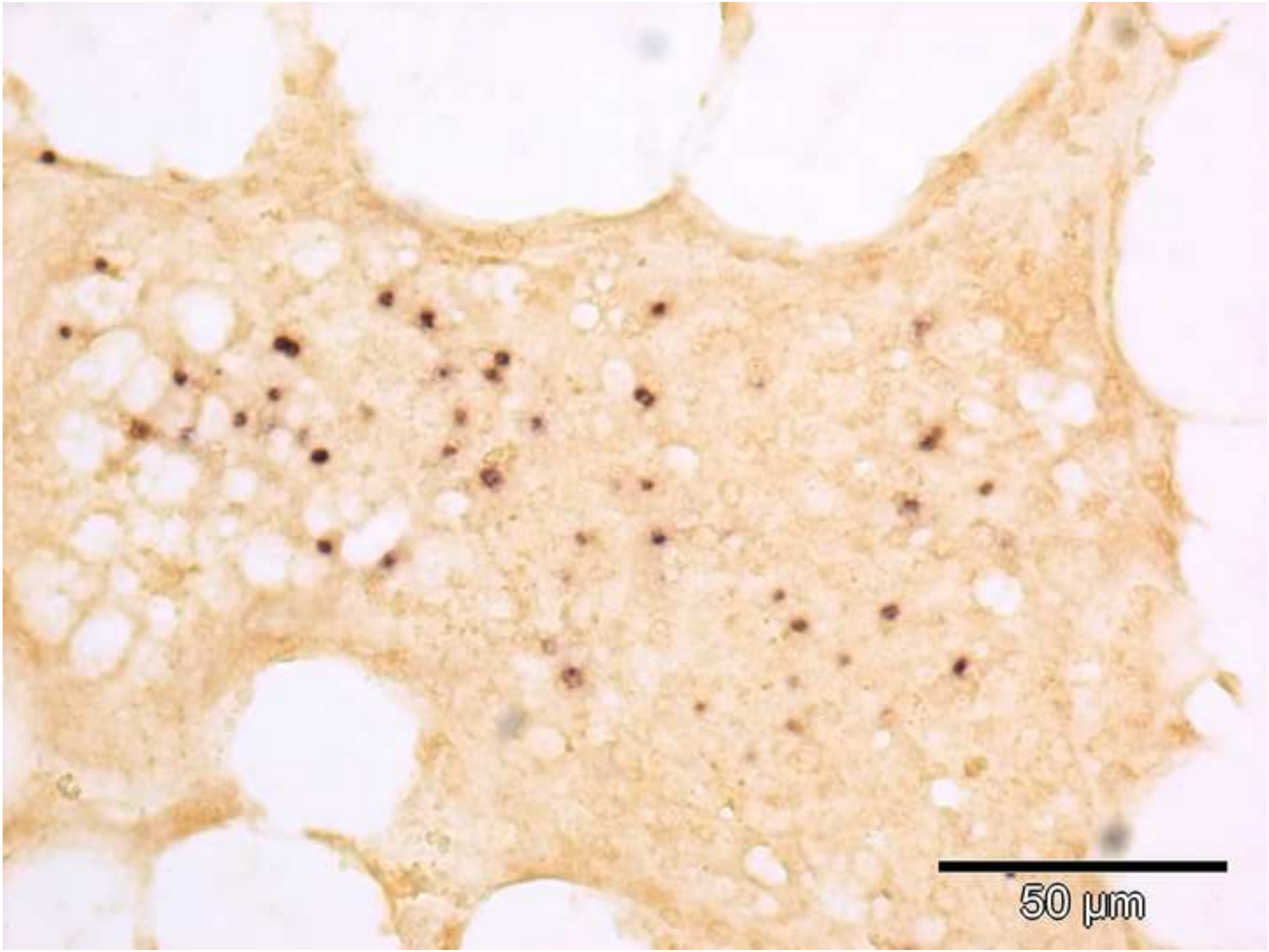

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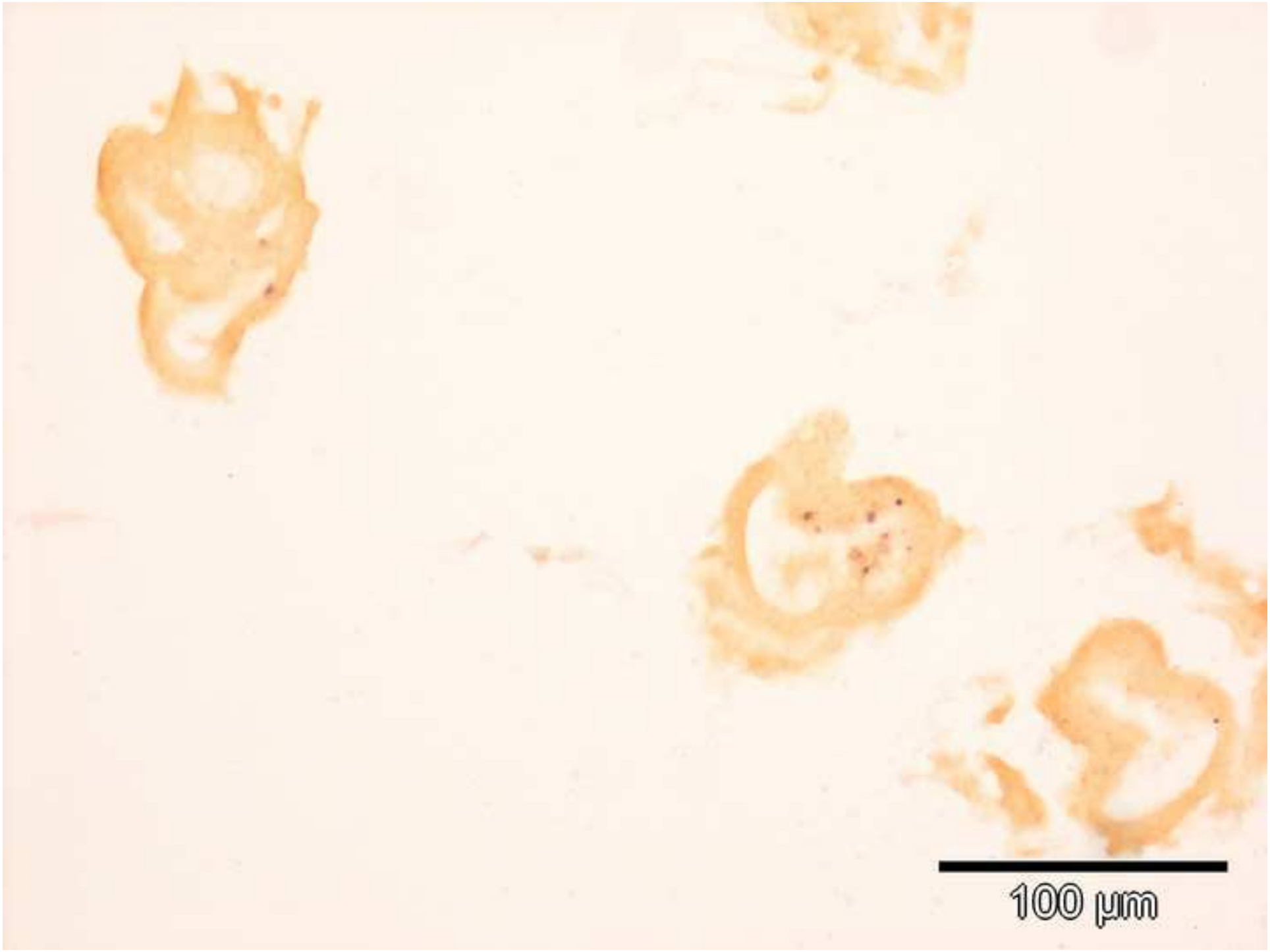

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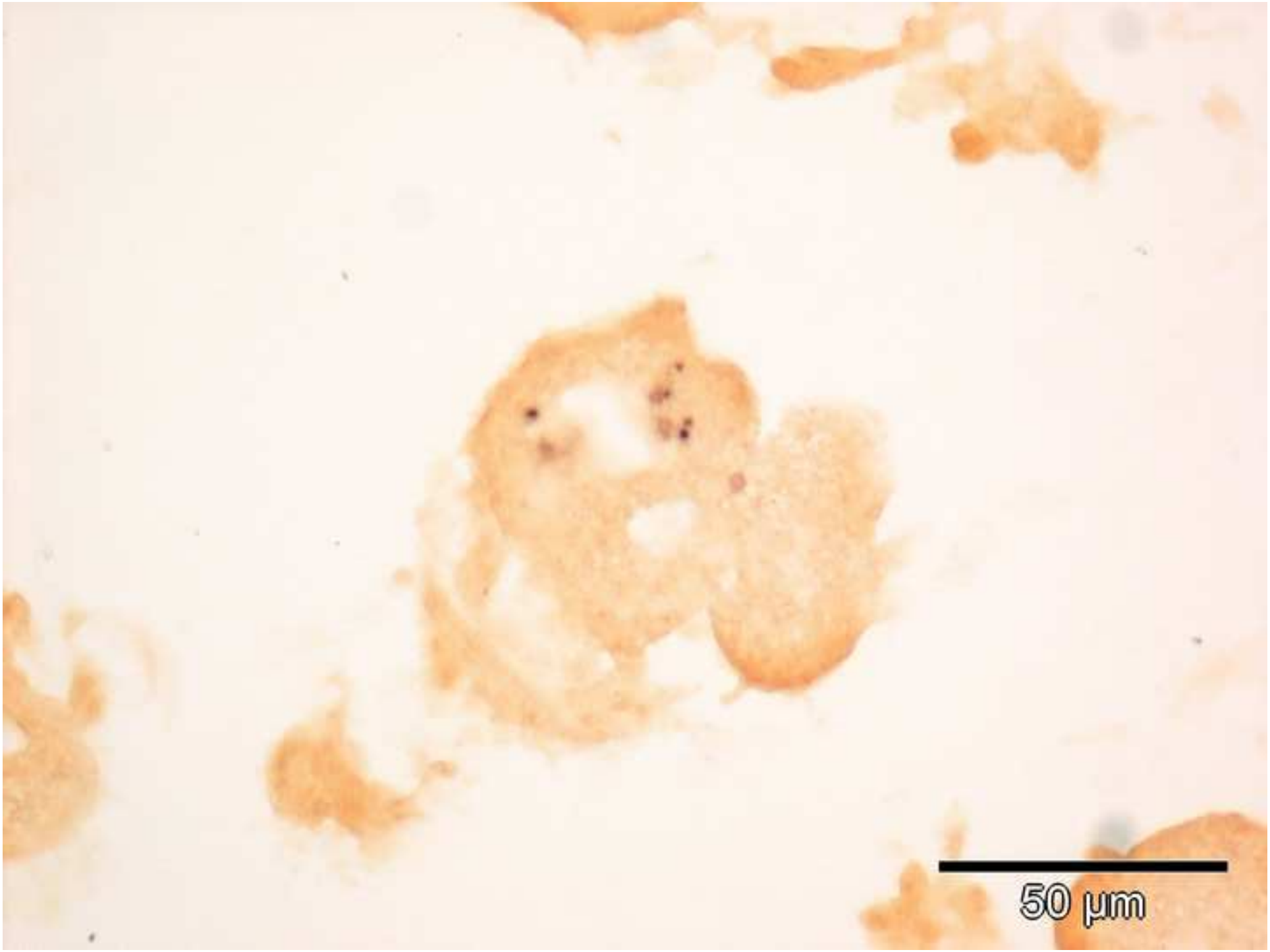

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