# Chapter 3

# Preparation of Diplonemid Samples for Microscopy

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## Abstract

This chapter provides a comprehensive set of protocols for preparing diplonemid cells for a range of microscopy applications, including live-cell imaging, fixed-cell fluorescence microscopy, endocytosis assay, immunofluorescence assay (IFA), and fluorescence in situ hybridization (FISH). It presents detailed instructions for slide preparation, cell harvesting, chemical fixation, permeabilization, and fluorescent labelling, with critical steps highlighted throughout. These protocols are designed to be adaptable for different experimental goals, enabling researchers to obtain high-quality imaging data for exploring the cellular biology of diplonemids.

**Key words** Live-cell imaging, Cell fixation, Cell permeabilization, Fluorescence staining, Fluorescence microscopy, Endocytosis assay, Immunofluorescence assay, Fluorescence in situ hybridization

## 1 Introduction

Microscopy plays a central role in diplonemid research, offering insights into their distinctive morphological features, physiological processes, and molecular organization. Routine light microscopy at low magnification provides an overall view of these microorganisms in their native state, helping to assess culture fitness and monitor cell motility. A more detailed observation of subcellular features of individual cells can be achieved using phase-contrast or, alternatively, differential interference contrast (DIC) microscopy, both enhancing the contrast of transparent diplonemid cells without the need for staining. However, the resolution limits of optical microscopy constrain our ability to study subcellular components. To overcome these limitations, transmission and scanning electron microscopies (TEM and SEM, respectively) are utilized to visualize ultrastructure and surface morphology at nanometer-scale resolution [1–3]. For instance, serial sectioning has enabled 3D reconstructions of the nucleus [4, 5], whereas serial block face SEM has

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helped to reveal a novel organelle in diplonemids [6]. While the aforementioned techniques uncover structural details, fluorescence and confocal microscopy allow tracking the spatial distribution of organelles, proteins, or other molecular targets within the cell [7, 8]. However, these microscopy techniques require meticulous sample preparation to minimize artifacts. Given that the only diplonemids currently available in culture are marine species, preparing samples to preserve their structure for the imaging can be challenging, largely due to their motility, sensitivity to osmotic stress, and generally poor adhesion to microscope slides, even when coated with common adhesive agents such as poly-L-lysine.

This chapter is focused on sample preparation for a range of visualization applications in diplonemid research. It presents an extensive set of protocols designed for both live- and fixed-cell imaging, covering techniques such as fluorescence staining, tracking of endocytic activity with fluorescent dextran, fluorescence in situ hybridization (FISH) for the detection of bacterial endosymbionts, and immunofluorescence assay (IFA) for protein localization. Many of these protocols were initially developed for the type species, *Paradiplonema papillatum*. However, unlike other diplonemids, this species has shown to be more resilient and is able to tolerate lower osmotic conditions. Accordingly, the protocols presented here have been adapted to ensure their applicability with other diplonemid species as well.

For live-cell imaging, we describe a strategy to immobilize motile cells without compromising their viability using low-gelling-temperature agarose, enabling the application of vital dyes that report on organelles and physiological states. For fixed-cell applications, we provide a general workflow encompassing fixation and permeabilization steps. Choosing a proper fixative is critical as it has a significant impact on preservation quality and compatibility with different imaging techniques. Formaldehyde and its polymerized form, paraformaldehyde (PFA), are commonly used to crosslink proteins and maintain cellular architecture with minimal autofluorescence, making them suitable for most applications. Glutaraldehyde provides superior ultrastructural preservation and is often used in electron microscopy, but its strong autofluorescence makes it unsuitable for fluorescence-based methods. In species where aldehyde fixation fails, osmium tetroxide can be used as an alternative. It reacts with lipids and proteins and can be applied either as a post-fixative for electron microscopy or as a standalone fixative. Accessing intracellular targets in fixed cells requires permeabilization, as many fluorescent dyes and antibody-based probes cannot penetrate intact cell membranes. In this chapter, we describe three permeabilization strategies routinely used for diplonemids: treatment with methanol, ethanol series, and a commercial permeabilization buffer.

## 2 Materials

### 2.1 Cell Harvesting

1. Laminar flow cabinet (if sterile conditions are required).
2. Falcon tubes (15–50 mL) for larger culture volumes, and/or microcentrifuge tubes (1.5–2 mL) for small samples.
3. Refrigerated centrifuges compatible with selected tubes.
4. Artificial seawater (ASW; 36 g/L) for washing and resuspension: Dissolve 36 g of anhydrous sea salts in 900 mL of deionized water in a 1 L beaker. Adjust the volume to 1 L in a graduated cylinder and filter through a sterile 0.22 μm filtration system into a sterile bottle.

### 2.2 Live-Cell Fluorescence Imaging

1. Live-cell fluorescent dyes (e.g., LysoTracker™ Green DND-26, NucBlue™ Live Cell Stain).
2. Artificial seawater (ASW; 36 g/L).
3. Microcentrifuge tubes (1.5 mL).
4. Aluminum foil to wrap microcentrifuge tubes.
5. Microcentrifuge.
6. 2% ultra-low gelling agarose: Add 0.2 g of agarose to 10 mL of ASW. Stir the mixture continuously at a rapid pace on a hotplate stirrer set to 70 °C and boil for 5 min to ensure the agarose fully dissolved (*see* **Note 1**). Allow the solution to cool to ~25 °C.
7. Microscope slides and cover slips (24 mm × 60 mm), or alternatively, 35 mm glass-bottom microscope dish.
8. Lightproof sample box.

### 2.3 Chemical Fixations for Fixed-Cell Imaging

1. Certified chemical fume hood.
2. Personal protective equipment (gloves, goggles, lab coat).
3. Hermetic containers for solid and liquid waste disposal.

#### 2.3.1 Aldehyde Fixation

*Formaldehyde and paraformaldehyde (PFA) fixation (for staining with fluorescent dyes and IFA)*

1. Artificial seawater (ASW, 36 g/L) or phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.4.
2. Formaldehyde-based fixative. To prepare a 1 mL of 4% formaldehyde solution, mix 250 μL of 16% methanol-free formaldehyde (e.g., Thermo Fisher) with 750 μL of ASW or PBS (for *P. papillatum*). If using a 37% formaldehyde (formalin), dilute 108 μL in 892 μL of ASW or PBS to obtain a final volume of 1 mL.

3. PFA-based fixative. To prepare a 4% PFA solution, add 0.8 g of PFA powder to 20 mL of ASW in a 50 mL Falcon tube. Incubate the tube in a 65 °C water bath, vortex occasionally to promote dissolution. Complete dissolution typically takes 1–1.5 h (*see* **Note 2**). Once dissolved, allow the solution to cool to room temperature (RT) before use. The fixative can be stored at 4 °C in the dark for up to 2 weeks or at −20 °C for several months.

*Glutaraldehyde fixation (for electron microscopy of P. papillatum)*

1. 2.5% (v/v) glutaraldehyde: Dilute 1 part of 25% aqueous glutaraldehyde solution in 9 parts of 0.2 M sodium cacodylate buffer. Protect from light. Store aliquots at −20 °C for short-term use.
2. Phosphate-buffered saline (PBS).

#### 2.3.2 Osmium-Based Fixation (for FISH, Post-Fixation Staining with Fluorescence Dyes)

1. 4% water-based $OsO_4$ (commercially available).
2. Graduated disposable plastic Pasteur pipettes.
3. 2× artificial seawater (ASW, 72 g/L).
4. Deionized water.

### 2.4 Cell Adhesion to Microscopy Slides

1. Adhesive microscope slides (e.g., poly-L-lysine-coated, Sigma). To prepare in-house adhesive gelatin-coated slides, soak standard microscope slides in a cleaning solution (2.5 M NaOH, 105 mL of absolute ethanol, 70 mL of ultrapure or distilled water) for 2 h. Meanwhile, prepare a 0.1% (w/v) gelatin solution by dissolving 0.2 g of gelatin in 200 mL of distilled water. Add 0.02 g of $CrK(SO_4)_2$, which corresponds to a concentration of 0.01% w/v. Heat the mixture to 65 °C on a magnetic hotplate stirrer, stirring continuously until the gelatin is completely dissolved. Afterward, filter the solution through a 0.22 μm filter. Rinse the cleaned slides thoroughly with distilled water, then dip them into the gelatin solution, and air dry. Store dried slides in a microscope slide box until use.
2. Pencil or oil-based resistant marker.
3. Blotting paper.
4. Fume hood.
5. Coplin jar or similar container.
6. *Optional*: liquid blocking PAP pen.

### 2.5 Permeabilization of Fixed Cells

1. Permeabilization agents:
   (a) 100% methanol (pre-chilled to −20 °C).
   (b) Commercial permeabilization buffer (e.g., eBioscience™ Permeabilization Buffer (10×), Invitrogen 00-8333-56).

To prepare 1× or 2× concentration, dilute 10× buffer with ultrapure water.

(c) 50%, 80%, 100% molecular biology grade ethanol (to prepare 50% and 80% concentrations, dilute 100% ethanol with ultrapure water).

2. 50 mL Falcon tube or similar sealable container.
3. Phosphate-buffered saline (PBS).

### 2.6 Processing Live Cells with Fluorescent Markers for Post-Fixation Imaging (for Staining with Fixable Fluorescent Dyes and/or Detecting Endogenously Expressed Fluorescence Signals)

1. Fixable fluorescent dyes (e.g., Hoechst 33342).
2. Phosphate-buffered saline (PBS).
3. Antifade DABCO mounting medium: 1% (w/v) 1,4-diazabicyclo[2.2.2]octane, 90% glycerol, 50 mM sodium phosphate, pH 8.0. *Optional*: add 100 ng/mL DAPI for nucleic acid staining.
4. Aluminum foil.
5. Microscope slides and cover slips (24 mm × 60 mm).
6. Lightproof storage box.

### 2.7 Fluorescence Staining of Fixed Cells

1. Fluorescent dyes (e.g., RedDot1, DAPI, SYBR Green, acridine orange).
2. Phosphate-buffered saline (PBS).
3. Antifade mounting medium: homemade DABCO mounting medium (*Alternative*: 50–90% (v/v) glycerol, 0.5% (w/v) N-propyl gallate, 20 mM Tris, pH 8.0) or commercial options (e.g., ProLong™ Gold Antifade Mountant, ProLong™ Diamond Antifade Mountant). Antifade mounting medium can be supplemented with nucleic acid stain, such as DAPI or Hoechst.
4. Aluminum foil.
5. Adhesive microscope slides and cover slips (24 mm × 60 mm).
6. Lightproof storage box.

### 2.8 Vital Staining of *P. papillatum* with Fluorescent Dextran (to Monitor Endocytosis)

1. Fluorescein isothiocyanate (FITC)-dextran (e.g., Sigma Aldrich). Prepare a 50 mg/mL stock solution.
2. Sterile artificial seawater (ASW, 36 g/L).
3. Phosphate-buffered saline (PBS).
4. Antifade mounting medium with nucleic acid stain.

### 2.9 Immunofluorescence Assay

1. 4% PFA solution, 100% methanol (for slide-based workflow), 1× commercial permeabilization buffer (for tube-based workflow).
2. Primary antibody.

3. Secondary antibody conjugated to fluorophore.
4. Phosphate-buffered saline (PBS).
5. PBS with 0.05% (v/v) Tween® detergent (PBS-T).
6. 5.5% FBS (v/v) in PBS-T or 5% (w/v) of skim milk powder for blocking and 3% (w/v) of bovine serum albumin (BSA) in PBS-T or 5% (w/v) of skim milk powder for antibody incubation.
7. Humid chamber (homemade option: fill a sealable plastic box with blotting paper, spray it with distilled water, and wrap the container with aluminum foil).
8. Parafilm.
9. Coplin jar or similar container.
10. Aluminum foil.
11. Adhesive microscope slides and cover slips (24 mm × 60 mm).
12. Antifade mounting medium with or without nucleic acid stain.
13. Lightproof storage box.

### *2.10 Fluorescence In Situ Hybridization (FISH) with Bacterial Eub338 Probe for Endosymbiont Screening*

1. Eub338 oligonucleotide probe (5′-labelled with fluorophores, such as FITC, Cy3, Cy5, ATTO, or Alexa Fluor dyes).
2. Hybridization oven with a horizontal shaker.
3. 50 mL Falcon tube or similar sealable container.
4. 3-ply tissue paper.
5. 5 M NaCl stock solution: Dissolve 29.22 g of NaCl in 80 mL of ultrapure water. When dissolved completely, adjust the volume to 100 mL. Store at RT.
6. 1 M Tris–HCl stock solution, pH 7.2: Dissolve 12.1 g of Tris base in 70 mL of deionized water. Adjust the pH to 7.2 by adding 10 N HCl dropwise while monitoring with a pH meter. Once adjusted, bring the volume up to 100 mL with deionized water. Store at 4 °C.
7. Hybridization buffer (900 mM NaCl, 20 mM Tris–HCl, 0.01% SDS) with 35% (v/v) formamide: In a 50 mL Falcon tube, add 10 mL of ultrapure water, 9 mL of 5 M NaCl, 1 mL of 1 M Tris–HCl (pH 7.2), 17.5 mL of deionized formamide, and 50 μL of 10% SDS. Fill up to 50 mL with ultrapure water and mix well. Store at 4 °C.
8. Washing buffer (225 mM NaCl, 20 mM Tris–HCl, 0.01% SDS): In a 0.5 L graduated cylinder, add 400 mL of deionized water, 7 mL of 5 M NaCl, 10 mL of Tris–HCl (pH 7.2), and 0.5 mL of 10% SDS. Fill up to 500 mL with deionized water, mix well and transfer to a 0.5 L bottle. Store at 4 °C. If SDS precipitates, warm the solution to 30–35 °C until it fully re-dissolves.
9. Parafilm.

10. Adhesive microscope slides and cover slips (24 mm × 60 mm).
11. Antifade mounting medium with nucleic acid stain (e.g., DAPI).
12. Lightproof storage box.

## 3 Methods

### 3.1 Cell Harvesting

For optimal viability and metabolic activity, collect diplonemids during the exponential growth phase, unless the experimental design specifically requires cells in a different physiological state. If diplonemids are to be cultured or processed under aseptic conditions, perform all sample handling within a laminar flow cabinet to ensure sterility.

1. Based on the cell density and required sample volume, transfer the appropriate amount of culture into a suitable microcentrifuge or Falcon tube and centrifuge at 3000 × *g* for 10 min (*see* **Note 3**) in a refrigerated centrifuge set to +15 °C (*see* **Note 4**).
2. Discard the supernatant without disturbing the pellet.
3. Gently resuspend the cells in ASW. If the culture was harvested in a Falcon tube, you may use a small volume (e.g., ~1 mL) of ASW to resuspend the cells and then transfer the suspension to a microcentrifuge tube.
4. Centrifugate the resuspended cells again under the same conditions to eliminate any residual components of culture medium, which could interfere with staining procedures.
5. Discard the supernatant. At this point, the cells are ready for downstream applications.

### 3.2 Live-Cell Fluorescence Imaging

1. After completing the standard harvesting procedure described in the previous subheading, resuspend the washed cell pellet in 1 mL of sterile ASW to obtain a uniform cell suspension (*see* **Note 5**).
2. Add the selected fluorescent dye(s) (*see* **Note 6**) directly to the cell suspension to achieve a final concentration recommended by the manufacturer (typically in the range of 0.1–1 μM for most commercial dyes). From this point onward, protect the sample from light to prevent photobleaching.
3. Gently mix the suspension by pipetting or inverting the tube to ensure even distribution of the dye throughout the cell population.

4. Incubate the cell-dye mixture at the recommended temperature (usually culturing temperature or RT) for 15–30 min, following the manufacturer's instructions.
5. After incubation, centrifugate the sample at 3000 × *g* for 10 min.
6. Discard the supernatant without disturbing the pellet.
7. To wash out any unbound dye, resuspend the pellet in 1 mL of sterile ASW and centrifuge again under the same conditions. Repeat the wash if necessary.
8. After the wash, resuspend the cells in 0.5 mL of ASW.
9. To immobilize the cells for microscopy, mix 0.5 mL of 2% ultra-low gelling agarose with an equal volume of the stained cell suspension (*see* **Note 7**). Immediately apply the agarose-cell mixture as a thin layer onto a microscope slide and place a coverslip on top, or alternatively, dispense the mixture into a microscope dish. Keep the slide or dish horizontal in a light-proof sample box to protect it from light until imaging.
10. As soon as the sample solidifies (*see* **Note 8**), proceed with fluorescence microscopy imaging using the appropriate filters for the selected dyes.

### 3.3 Chemical Fixations for Fixed-Cell Imaging

***Warning:*** Handle chemical fixatives and perform all fixation steps in a certified chemical fume hood while wearing appropriate personal protective equipment, including gloves, goggles, and a lab coat. When working with osmium tetroxide ($OsO_4$), it is advisable to wear double gloves and to protect surfaces with absorbent paper. Strictly follow safety regulations and dispose solid and liquid waste according to established safety protocols.

#### 3.3.1 Aldehyde Fixation

*Formaldehyde and paraformaldehyde (PFA) fixation (for staining with fluorescent dyes and IFA)*

1. Harvest cells as described in Subheading 3.1 and, depending on the size of the pellet, resuspend them in 250–500 µL of the 4% fixative. Mix gently until a homogenous suspension is obtained.
2. For slice-based IFA assay (that we recommend for *P. papillatum*), spread the cells immediatelly on the microscopy slides, incubate at RT for 15 min and after fixation rinse the cells with PBS.
3. For tube-based workflow, incubate the cells in the tubes for 15–30 min and after fixation, centrifuge the sample at 1500–2000 × *g* (*see* **Note 9**) for 10 min at RT. Discard the supernatant into a designated waste container, then resuspend the pellet in 200–500 µL of either distilled water (FISH,

fluorescence staining) or PBS (IFA, fluorescence staining). Repeat the wash once more under the same conditions.

*Glutaraldehyde fixation (for electron microscopy of P. papillatum)*

1. Prepare cell pellet as described in Subheading 3.1 and carefully resuspend it in 2.5% glutaraldehyde. The volume of fixative should be sufficient to completely cover the pellet (typically 250–500 μL, depending on pellet size).
2. Incubate the suspension for 1 h at RT or up to several hours at 4 °C.
3. Following fixation, pellet the cells again at 1500–2000 × *g* for 10 min and carefully discard the supernatant into a designated waste container.
4. Wash the fixed cells twice in sterile PBS (or 0.2 M sodium cacodylate buffer) to remove residual glutaraldehyde. For each wash, gently resuspend the pellet in 500 μL buffer, centrifuge, and discard the supernatant.

#### *3.3.2 Osmium-Based Fixation (for FISH, Post-Fixation Staining with Fluorescent Dyes)*

1. Prepare a fresh 2% (v/v) $OsO_4$ working solution immediately before use (*see* **Note 10**). Using a graduated disposable plastic Pasteur pipette, mix 100 μL of 4% aqueous $OsO_4$ solution with 100 μL of 2× ASW (7.2% w/v). Mix thoroughly by pipetting.
2. Harvest cells as described in Subheading 3.1, leaving ~20 μL of fluid. Resuspend the cell pellet in this residual volume.
3. Carefully add 200 μL of 2% $OsO_4$ solution to the resuspended cells using a Pasteur pipette and mix well. Ensure not to spill the $OsO_4$ and do not allow it to come into contact with the exterior of the microcentrifuge tube.
4. Incubate the cell suspension for 15 min at RT.
5. Pellet the fixed cells by centrifugation at 1500–3000 × *g* for 10 min. Discard the supernatant into a designated osmium waste container, following hazardous waste disposal guidelines.
6. Resuspend the pellet in 1.2 mL of distilled water, centrifuge, and discard the supernatant. Repeat this washing step four times to ensure complete removal of osmium residues. Finally, resuspend the pellet in 200–500 μL of distilled water.

### *3.4 Cell Adhesion to Microscopy Slides*

1. Label an adhesive microscope slide using a pencil or oil-based resistant marker. *Optional*: draw a hydrophobic barrier using a liquid-blocking PAP pen to confine the sample area.
2. Apply a large drop (~200 μL) of the fixed cell suspension to the middle of the slide and spread it evenly using a pipette tip.

3. Allow the cells to settle and adhere for 15 min at RT.
4. Carefully remove the excess liquid by gently touching the top of the droplet with blotting paper, avoiding disturbance to the cells.
5. Transfer the slide to a fume hood and let any residual liquid to evaporate completely. It is crucial not to overdry the cells. Monitor the slide, and as soon as the liquid has evaporated, immediately proceed to **step 6**.
6. Thoroughly rinse the slide by dipping it into a jar with deionized water, then dry it by tilting and gently tapping onto a tissue paper.

### 3.5 Permeabilization of Fixed Cells

#### 3.5.1 With 100% Methanol for Staining with Fluorescent Dyes and IFA

Place the slide with adhered cells into a 50 mL Falcon tube or a similar container filled with 100% methanol pre-chilled to −20 °C or spread 400μL of 100% methanol directly on the slide (recommended for slice-based IFA assay of *P. papillatum*). Incubate the sample for 15–20 min at RT. After incubation, remove the slide from methanol and rinse it with PBS (*see* **Note 11**). To remove excess PBS, tilt the slide and gently tap it against tissue paper, avoiding direct contact with the cell area to prevent detachment or damage.

#### 3.5.2 With Commercial Buffer for Staining with Fluorescent Dyes and IFA

Permeabilization can be performed either directly in suspension or on slides with affixed cells. To permeabilize in suspension, fix the cells as described in **steps 1** and **2** of Subheading 3.3.1 *Formaldehyde and PFA fixation*. Next, add an equal volume of 2× permeabilization buffer directly to the cell suspension. Centrifugate the sample at 1500–2000 × *g* for 10 min at RT. Discard the supernatant into a designated waste container and resuspend the pellet in 200–500 μL of 1× permeabilization buffer. Continue with downstream application or proceed with cell adhesion as described in Subheading 3.4. To permeabilize cells that have already been fixed and adhered to slides, apply 250 μL of 1× permeabilization buffer directly onto the slide, incubate for 10 min at RT, and then wash thoroughly with PBS or distilled water.

#### 3.5.3 With Ethanol for FISH and DAPI Staining

Sequentially apply 50%, 80%, and 100% ethanol directly onto the horizontally positioned slide, incubating with each concentration for 3 min. Between each step, remove excess ethanol by tilting the slide and tapping it onto tissue paper.

Sample slides not processed immediately for downstream applications can be stored in 100% ethanol or methanol at −20 °C in sealed Falcon tubes or jar for several days.

### 3.6 Processing Live Cells with Fluorescent Markers for Post-Fixation Imaging (for Staining with Fixable Fluorescent Dyes and/or Detecting Endogenously Expressed Fluorescence Signals)

1. Harvest cells as described in Subheading 3.1. If the experiment involves only fluorescent protein-expressing cells (e.g., YFP-tagged [9]), and no live staining, proceed directly to **step 3**.
2. If using a fixable fluorescent dye(s), add the dye to the suspension of live cells at the manufacturer's recommended working concentration. Incubate in the dark for the specified time (typically 10–30 min) at RT or as instructed. Centrifuge at 1500–2000 × *g* for 10 min, discard the supernatant, and resuspend the pellet in PBS.
3. Fix the cells using a formaldehyde-based fixative as described in Subheading 3.3.1 *Formaldehyde and PFA fixation*.
4. Resuspend the fixed cells in a small volume (~10 μL) of antifade DABCO mounting medium with or without DAPI, depending on the experimental requirements.
5. Mount the suspension onto a microscope slide and place a coverslip on top, avoiding air bubbles. Store the slides at 4 °C in the dark until imaging.

### 3.7 Fluorescence Staining of Fixed Cells

1. Harvest cells as described in Subheading 3.1 and fix them using an appropriate protocol described in Subheading 3.3.
2. Allow the cells to adhere to slides either before or after staining, depending on whether the staining will be performed in suspension (in tubes) or on the slide surface (*see* Subheading 3.4). Add the fluorescent dye(s) to either the cell suspension or directly onto the cells that have adhered to the slides (*see* **Note 12**) at the recommended working concentration. Incubate in the dark for the specified time (typically 10–30 min) at RT or as instructed by the manufacturer.
3. After staining, rinse the cells by dipping slides in PBS (at least two times) to remove unbound dye. For suspended cells, centrifugate at 1500–2000 × *g* for 10 min, discard the supernatant, and then resuspend the pellet in PBS or distilled water.
4. If not already adhered, allow the stained cells to attach to adhesive microscope slides as described in Subheading 3.4.
5. Apply a drop of antifade mounting medium with or without nucleic acid stain, depending on the experimental requirements, and place a coverslip on top, avoiding air bubbles. Store the slides at 4 °C in the dark until imaging.

### 3.8 Vital Staining of *P. papillatum* with Fluorescent Dextran (to Monitor Endocytosis)

1. Collect 1 mL of an exponentially growing *P. papillatum* culture as described in Subheading 3.1. Resuspend the cell pellet in sterile ASW and incubate for 15 min at RT to enhance endocytic activity.

2. Add 50 mg/mL of FITC-dextran stock solution to the cells to achieve a final concentration of 5 mg/mL (e.g., mix 100 µL of the cell suspension with 10 µL of FITC-dextran stock).
3. Incubate the cells with FITC-dextran for a selected time point (e.g., 1, 2, 5, 15, or 30 min) at RT, depending on the experimental objective. From this point on, protect the samples from light.
4. After incubation, centrifugate the cells at 1500–2000 × *g* for 10 min, discard the supernatant to remove excess dextran, and then resuspend the pellet in PBS (*see* **Note 13**).
5. Fix the cells in 4% PFA as described in Subheading 3.3.1 *Formaldehyde and PFA fixation* and allow them to adhere to microscope slides following the procedure in Subheading 3.4.
6. Apply a drop of antifade mounting medium over the sample area and place a coverslip on top, avoiding air bubbles. Store the prepared slides at 4 °C in the dark until imaging.
7. Visualize the cells using a fluorescence microscope equipped with filters appropriate for FITC and DAPI detection.

### 3.9 Immunofluorescence Assay

**Tube-Based Workflow:**

Note that processing in tubes may lead to cell loss during centrifugation.

1. Harvest cells (> 2 × $10^6$/sample) as described in Subheading 3.1.
2. Fix the cells with 4% PFA following **steps 1** and **2** in Subheading 3.3.1 *Formaldehyde and PFA fixation*.
3. Permeabilize the cells using a commercial buffer, as described in Subheading 3.5 for cell suspension.
4. Resuspend the permeabilized cells in 200–500 µL of 1× permeabilization buffer (*see* **Note 14**) and add the primary antibody at the recommended concentration. Incubate for 2 h or overnight at 4 °C.
5. After incubation, centrifugate the cells at 1500–2000 × *g* for 10 min at RT. Discard the supernatant, resuspend the pellet in distilled water, and repeat the centrifugation. Repeat the wash step twice.
6. Dilute the secondary antibody in 1× permeabilization buffer to the appropriate concentration and add it to the washed cell pellet. Incubate for 1 h at RT in the dark.

7. Wash the cells as described in **step 3** and resuspend in 200 μL of PBS or ultrapure water. Transfer 30 μL of the cell suspension to the microscopy slide and allow to adhere as instructed in Subheading 3.4, protecting from light (*see* **Note 15**).
8. Proceed to the mounting steps described below.

**Slide-Based Workflow:**

1. Harvest cells and fix them with 4% PFA according to Subheadings 3.1 and 3.3.1 *Formaldehyde and PFA fixation*, respectively. Allow cells to attach to slides as directed in Subheading 3.4, then permeabilize with methanol as described in Subheading 3.5.
2. Place the slide in a humid chamber and apply 250 μL of 5.5% (v/v) FBS in PBS-T or 5% (w/v) milk to block non-specific antibody binding. Cover the slide with parafilm and incubate at RT for 45 min.
3. Remove the parafilm and rinse the slide gently using PBS.
4. Apply 250 μL of primary antibody diluted to the appropriate concentration in 3% BSA in PBS-T or 5% milk. Cover with parafilm and incubate in a humid chamber overnight at 4 °C.
5. Remove the parafilm and wash the slide three times gently using PBS-T and twice with PBS to eliminate unbound antibody.
6. Apply 250 μL of secondary antibody diluted to the appropriate concentration in 3% BSA in PBS-T or 5% milk. Cover with parafilm and incubate at RT for 1 h in the dark.
7. Remove the parafilm and wash the slide three times using PBS-T and twice using PBS.
8. Tilt the slide and gently tap its edge onto a tissue paper to remove PBS.

**Mounting:**

9. Apply a drop of antifade mounting medium with or without nucleic acid stain, depending on experimental requirements, and place a coverslip on top, avoiding air bubbles.
10. Store the prepared slide at 4 °C in the dark until imaging.

### *3.10 Fluorescence In Situ Hybridization (FISH) with Bacterial Eub338 Probe for Endosymbiont Screening*

In addition to the experimental slide, prepare both positive and negative controls. If an endosymbiont-bearing diplonemid is not available, any free-living bacterial species possessing a 16S rRNA region complementary to the Eub338 can be used as a positive control. The negative control is a slide of the diplonemid species in question, incubated under identical conditions but without the Eub338 probe.

1. Harvest diplonemid cultures as described in Subheading 3.1.
2. Fix cells with 4% PFA according to Subheading 3.3.1 *Formaldehyde and PFA fixation* and adhere them to microscopy slides following Subheading 3.4.
3. Permeabilize the affixed cells as described in Subheading 3.5.3.
4. Preheat the hybridization oven to 46 °C.
5. Thaw the Eub338 probe on ice, protecting it from light (*see* **Note 16**).
6. Prepare hybridization vessels by placing a folded, 3-ply tissue paper strip at the bottom of a 50 mL Falcon tube positioned horizontally. Spread 1.5–2 mL of hybridization buffer onto the tissue until it is fully soaked, then seal the tube until use.
7. For each slide, prepare 250 μL of hybridization buffer containing 250 nM Eub338 probe. Apply this solution onto the center of a dry slide (ensure no residual ethanol remains) with the fixed cells. Immediately cover the sample area with parafilm, avoiding air bubbles and ensuring complete coverage. From this point on, minimize the exposure of slides to light.
8. Place the slide above the soaked tissue paper inside the hybridization vessel and close securely.
9. Place the vessel in the hybridization oven and incubate at 46 °C for 1.5 to 3 h. During the incubation, ensure that the slides are completely protected from light.
10. For each slide, prepare a Falcon tube containing 50 mL of washing buffer, preheated to 48 °C in a water bath. After hybridization, remove the slide from the vessel, discard the parafilm, and quickly rinse with ~10 mL of warm washing buffer.
11. Immerse the slides inside the Falcon tubes with remaining washing buffer. Place the tubes in the oven on a horizontal shaker set to 50–60 rpm and incubate at 48 °C for 25 min in the dark.
12. Briefly rinse the slide by dipping it into deionized water, then allow to air-dry in the dark.
13. Mount the sample by applying two drops of antifade mounting medium and place a coverslip, avoiding air bubbles. Store the slide at 4 °C in the dark until imaging.

## 4 Notes

1. The temperature and boiling time for preparing agarose may vary depending on the specific type and brand of agarose used. To streamline the overall workflow, agarose preparation can be

started in parallel with cell incubation and washing (**steps 4–8** of Subheading 3.2). However, be careful not to overheat the agarose solution, as this can degrade its gelling properties.

2. Add 2 M NaOH dropwise to aid paraformaldehyde dissolution, as it requires an alkaline environment to fully dissolve.
3. A centrifugation speed of 3000 × *g* is sufficient to pellet cells of most cultured diplonemids without causing mechanical stress or compromising cell integrity. After centrifugation, you may inspect the supernatant for residual cells and the pellet for cell integrity under the microscope. If cells remain in the supernatant or the pellet appears loose or damaged, adjust the centrifugation speed and/or time. For larger cells, consider reducing the centrifugation speed to 1000–2000 × *g* to minimize cell damage. Due to the poor adhesion of *P. papillatum* to miscroscopy slides, we recommend to spin down at least 20–30 mL of exponentially growing cells for slide-based IFA method, while for tube-based workflow, about 5–10 mL of diplonemid culture is usually sufficient.
4. For species cultivated at a different temperature, set the centrifuge accordingly.
5. Ensure that the cell density is suitable for imaging. Typically, a concentration of 1–5 × $10^6$ cells/mL is recommended for optimal visualization without overcrowding.
6. Fluorescence dyes are often supplied as ready-to-use stock solutions; however, some may require dilution in appropriate solvents. If needed, prepare a fresh stock solution according to the manufacturer's instructions. Ensure that the dye is fully dissolved before use, and protect it from light to prevent photobleaching.
7. When combining the agarose and stained cells, it is essential to add the cell suspension to the agarose, not the other way around. This minimizes the risk of premature gelling and ensures even distribution. Mix quickly and gently to avoid clumping.
8. The agarose-cell mixture will solidify within seconds to minutes after application, depending on the layer's thickness and temperature. If the gelling temperature of the agarose ranges between 8 °C and 17 °C, the sample can be cooled for a few minutes in a refrigerator or on ice for the solidification process.
9. Fixed cells are more fragile and require moderate centrifugation.
10. The fixative must be freshly prepared just before use. Long-term storage of 4% $OsO_4$ stock solution must follow institutional safety regulations, including refrigeration in sealed, labelled containers inside secondary containment.

11. Depending on the downstream application, slides may be rinsed briefly or rehydrated by applying PBS dropwise or dipping the slide in PBS for up to 10 min, respectively.
12. For longer incubation periods, cover the slide with Parafilm and place it in a humid chamber.
13. *P. papillatum* is the only known diplonemid species that tolerates PBS in a live state; other marine diplonemids burst due to osmotic shock.
14. No blocking step is required when using commercial permeabilization buffer.
15. The remaining resuspended cells can be stored in the dark at 4 °C, and additional slides can be prepared as needed.
16. Minimize probe exposure to light throughout the procedure.

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