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A Practical Approach to Study Uncultivated Protists Using Single-Cell Techniques for Electron Microscopy

Maia V. Palka¹  | Kevin C. Wakeman^{2,3} | Eunji Park^{1,4}  | Gordon Lax^{1,5}  | Brian S. Leander^{1,4} 

¹Department of Botany, Beaty Biodiversity Research Centre and Museum, University of British Columbia, Vancouver, British Columbia, Canada | ²Institute for the Advancement of Higher Education, Hokkaido University, Sapporo, Hokkaido, Japan | ³Graduate School of Science, Hokkaido University, Sapporo, Hokkaido, Japan | ⁴Department of Zoology, Beaty Biodiversity Research Centre and Museum, University of British Columbia, Vancouver, British Columbia, Canada | ⁵Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada

Correspondence: Maia V. Palka (maia.palka@gmail.com) | Kevin C. Wakeman (wakeman.k@oia.hokudai.ac.jp)

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ABSTRACT

Protists represent a significant portion of eukaryotic diversity with a wide range of ecological roles, lifestyles, and diverse morphological traits. Despite their widespread importance in ecological systems and their potential as model organisms, most protist lineages remain poorly characterized, in part due to their small size and the fact that many lineages remain uncultivated. High-resolution microscopy techniques like transmission and scanning electron microscopy (TEM and SEM) are powerful tools for studying protists, but the application of these techniques has significant limitations when applied to uncultivated species, largely due to the absence of reproducible methodologies tailored to studying single cells with small sample sizes. We present a robust protocol for preparing individual eukaryotic cells for TEM and SEM that addresses these limitations, can be implemented in the field, and uses inexpensive and easily obtainable materials. Our method minimizes cell loss during sample preparation for TEM and SEM, and it enables the tracking of single cells through TEM preparation to ensure cells can be localized and oriented appropriately for ultramicrotome sectioning. This protocol expands the feasibility of ultrastructural studies on uncultivated protist lineages and aims to make high-resolution microscopy more accessible for the broader community of protistologists who study uncultivated taxa.

1 | Introduction

Protists encompass a significant proportion of eukaryotic diversity with a wide range of lifestyles, functions, behaviors and morphological traits. However, only a small fraction of this diversity has been studied in detail, especially using molecular and ultrastructural approaches (Pawlowski et al. 2012). While protists can vary significantly in size relative to one another, the majority are microscopic and therefore not visible with the naked eye (Burki et al. 2021). In fact, cells of some taxa, such as the chlorophyte alga *Ostreococcus tauri* and some members of the group Picozoa, can be less than 1 µm in size and have a significant overlap in size with many bacteria (Keeling 2007; Seenivasan et al. 2013). While some incredibly large protists (e.g., members of the foraminiferan clade Xenophyphorea, or

some phaeodarians) are visible to the naked eye, these represent outliers, as the majority of protists are microscopic single cells (Burki et al. 2021; Levin and Rouse 2020).

The small size of most protists poses a significant challenge to their study, and consequently, protistological research has historically been heavily reliant on cultivation (del Campo et al. 2024; Keeling 2019; Schoenle et al. 2025). Not only can the application of molecular techniques be challenging in the absence of cultivated taxa due to the small amounts of DNA/RNA in single cells (Zhou et al. 2020), but descriptive studies of most protists also require the application of a suite of high-resolution microscopy techniques, such as electron microscopy, for morphological characterization (Keeling 2019; Leander 2004). Studying protists using electron microscopy is essential for discovering novel

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morphological features and understanding the evolution of ecologically or functionally significant traits (Paulin 1990). When descriptive morphological studies are integrated with molecular characterizations of microbial eukaryotes, these data can address fundamental biological questions about trait evolution in deep-branching lineages or the genetic basis of key functional traits (Keeling 2019; Leander 2004).

1.1 | Electron Microscopy on Uncultivated Protists

Despite the remarkable morphological, molecular and phylogenetic diversity of microbial eukaryotes, most described and novel protist lineages are not cultivated and are therefore poorly studied, or restricted to single-cell techniques or indirect molecular approaches (i.e., environmental DNA surveys) (Burki et al. 2020, 2021; Keeling and Del Campo 2017). As can be imagined, working with uncultivated single-celled organisms has significant limitations in the techniques that can be applied and the range of biological questions that can be asked about these taxa. Significant advances in culture-independent molecular biology approaches have been made over the last 15 years, including the development of single-cell transcriptomics approaches (e.g., SmartSeq2, Picelli et al. 2014) and even molecular biology kits provided by manufacturers intended for the whole genome amplification of single cells (e.g., Qiagen REPLI-g Single-Cell Kit) (Heywood et al. 2011; Lyu et al. 2023; Picelli et al. 2014; Stepanauskas 2012; Troell et al. 2016). These methods are becoming increasingly represented in the protistological research. For example, single-cell transcriptomics have been used to answer important questions about deep phylogenetic relationships of poorly understood taxa (Lax et al. 2018) or the evolution of organellar genomes in dinoflagellates (Cooney et al. 2024), among many others. Approaches to generating ultrastructural data from single cells using electron microscopy have not kept up with these advances in molecular biology. Though some attempts have been made to describe electron microscopy sample preparation techniques for single cells (Truby 1997), most of these approaches lack sufficient detail and are not necessarily reproducible or adaptable to diverse lineages of eukaryotes and their often small sample sizes.

Ultrastructural studies of biological organisms rely on the effective fixation and dehydration of the biological specimen of interest to preserve the natural state of the organism and prepare it for viewing under an electron beam (Hayat 2000). This involves incubating specimens in various fixatives and buffers and requires the frequent exchange of solutions around the sample. When electron microscopy techniques are applied to animal or plant tissue, the exchange of liquids and fixatives is straightforward because specimens can be observed with the naked eye (Dykstra and Reuss 2003). There is minimal risk of specimen loss during these steps, and samples can even be manipulated and repositioned during the preparation process (e.g., positioning a specimen before embedding in an epoxy resin for transmission electron microscopy). Unfortunately, this is not the case when preparing individual microscopic organisms, such as protists and meiofaunal animals, because they can accidentally be resuspended and removed during liquid exchanges. Electron microscopy often requires the use of toxic fixatives and buffers (including aldehydes, osmium tetroxide, and sodium cacodylate

buffer) (Dykstra and Reuss 2003; Hayat 2000), which restricts the ability to perform solution exchanges under a stereoscope or inverted light microscope due to toxic fumes, unless they are installed in a fume hood. For this reason, microbial eukaryotes cannot be easily observed or tracked during preparation for electron microscopy, thereby making specimen loss difficult to prevent. As a result, ultrastructural studies of microbial eukaryotes using electron microscopy are often limited to cultured organisms that can be concentrated onto a membrane or into a pellet via centrifugation for processing.

1.2 | Challenges With Single-Cell Transmission Electron Microscopy (TEM)

TEM is used to visualize the internal ultrastructure of a biological specimen at an incredibly high magnification and resolution (Dykstra and Reuss 2003; Hayat 2000). Sample preparation involves a wet lab component that relies on the incubation and exchange of multiple different solutions surrounding the specimen of interest. This includes: (1) the fixation of specimens using glutaraldehyde which crosslinks proteins, or through high pressure freezing; (2) post-fixation using osmium tetroxide which interacts with lipid membranes and increases the electron density of organic tissues, thereby acting as a stain; (3) dehydration using a graded series of solvents (often, ethanol and acetone solutions); (4) the infiltration and embedding of samples in an epoxy resin, such as Spurr's resin or Epon 812. Each wet lab step involves multiple solution exchanges that can result in cell loss when specimens cannot be tracked by eye, such as in the case of microscopic organisms like protists. While this is an insignificant issue when applying TEM to large quantities of cultivated organisms, some studies that aim to describe the ultrastructure of uncultivated protists can have a sample size as small as one cell; thus, loss of cells during preparation steps can be a big concern.

Once samples are embedded in resin, they must be prepared for ultramicrotomy and ultrathin sections mounted on grids prior to visualization using a transmission electron microscope (Dykstra and Reuss 2003; Hayat 2000). Given that the total magnification for a stereoscope or ultramicrotome typically ranges from 40 to 60X depending on the microscope and eyepieces used, identifying a single cell prepared for TEM (especially for cells smaller than $\sim 50\mu\text{m}$) within a block of resin is at best arduous and often nearly impossible (He et al. 2006). On top of that, it is difficult to verify whether cell loss occurred during sample preparation of protists because cells are rarely processed within a container that can be observed using light microscopy beyond a stereoscope (which is unsuitable for observing most protists due to their small size). The high risk of cell loss and difficulty locating individual cells within a resin block often makes TEM unreliable for studying uncultivated protists, especially given the absence of a comprehensive published protocol specifically optimized for single cells.

Ultrastructural studies using TEM require adequate positioning of a sample within the resin block in order to obtain sections from a particular region of interest within the organism studied. Samples prepared for TEM are often embedded in epoxy resin using embedding capsules (e.g., BEEM capsules) or molds. While these are excellent choices for embedding

larger specimens that can be manipulated and positioned within a mold or capsule so that they can be sectioned from the appropriate orientation, these are not necessarily the most appropriate choice for embedding single cells. Because the majority of protists cannot be seen with the naked eye, it can be impossible to manipulate/reposition a single protist cell within a BEEM capsule, especially when embedding cells in an epoxy resin that needs to be used within a fume hood. With this in mind, in order to obtain specific sections through a protist for TEM with precision (such as cross sections), they need to be embedded in a way where the polymerized resin block containing cells can be positioned in any orientation for ultramicrotomy (Reymond and Pickett-Heaps 1983). This can be accomplished through flat-embedding, where samples are embedded in a thin layer of resin that can be later repositioned for sectioning.

Even without sample loss, TEM is susceptible to a plethora of issues that can influence image quality and data interpretation, including sample preparation artifacts and limited contrast in biological specimens (Dykstra and Reuss 2003; Montanaro et al. 2016). Sample preparation artifacts, when using chemical fixatives, can be caused by inadequate fixation (e.g., if specimens cannot be fixed immediately upon collection or if fixation is attempted using degraded fixatives), temperature fluctuations, and effects of pH and osmolarity changes when using unbuffered and non-isotonic fixatives (Brubacher et al. 2014; Montanaro et al. 2016). High-pressure freezing (HPF) is a physical fixation technique that serves as an alternative to chemical fixation as it can effectively minimize artifacts associated with chemical fixatives (Livingston et al. 2023; Montanaro et al. 2016). However, HPF is challenging to apply to single cells and is not possible in the field, thereby constraining its feasibility for the preservation of uncultivated protists.

The pH and osmolarity of fixative solutions are especially important to consider (Brubacher et al. 2014; Montanaro et al. 2016; Truby 1997). Glutaraldehyde fixation is pH-dependent; therefore, a drastic change in pH due to the oxidation of glutaraldehyde during sample fixation can influence the effectiveness of the fixation (Rasmussen and Albrechtsen 1974). Glutaraldehyde solutions are often buffered using sodium cacodylate or a phosphate buffer; however, there are disadvantages to the use of either of these buffers (Dykstra and Reuss 2003; Montanaro et al. 2016). Sodium cacodylate is toxic and must be handled carefully under a fume hood as it contains arsenic (thus limiting its use in the field). At the same time, though often non-toxic, phosphate buffers can react with osmium tetroxide (an essential post-fixative to provide contrast during imaging) to form precipitates that result in electron-dense artifacts in TEM images.

Fixation artifacts, such as swelling or shrinkage, in specimens prepared for TEM can also occur as a result of osmotic imbalances caused by differences between the native osmolarity of the specimen and the fixative solution; thus, it is important that the osmolarity of the fixative solution is adjusted to closely match the osmolarity of the environment from which the specimen originates (Dykstra and Reuss 2003; Montanaro et al. 2016). Unfortunately, many previous ultrastructural

studies of marine organisms use fixatives that are prepared using seawater and thus do not account for any change in the osmolarity or pH of the solution upon the addition of glutaraldehyde (Montanaro et al. 2016). When fixatives are prepared using a buffer or solvent that has a low osmolarity (e.g., sodium cacodylate), tonicity is often adjusted through the addition of compounds, such as sucrose, glucose, and salt (Hayat 2000; Montanaro et al. 2016).

Given the many chemical factors that can influence the quality of ultrastructural preservation of tissue, especially with marine organisms that can be particularly sensitive to osmotic imbalances, some effort has been described in the literature to determine optimal conditions for the fixation of various organisms. In fact, Montanaro et al. (2016) compared the fixation quality of marine invertebrates using fixatives prepared in filtered sea water, sodium cacodylate buffer, phosphate buffered saline and PHEM buffer, with the osmolarity of each fixative adjusted to match the osmolarity of sea water using sucrose. PHEM buffer contains PIPES and HEPES, both zwitterionic buffers that facilitate cross-linking and preserve lipid membranes, and EGTA, a chelating agent that promotes the fixation of microtubules (Baur and Stacey 1977; Hayat 2000; Montanaro et al. 2016; Schliwa and van Blerkom 1981). Montanaro et al. (2016) demonstrated that the ultrastructural preservation of marine invertebrates considerably improves when specimens are fixed using glutaraldehyde prepared in PHEM buffer compared to any other buffer. Given the demonstrated success of PHEM buffer in preserving the ultrastructure of marine invertebrates, and its accessibility for use in the field (given that it is non-toxic), it is an excellent candidate as a buffer for glutaraldehyde fixation in marine protists. The TEM images we present in this protocol are of an uncultivated marine euglenid *Dinema* sp., which was prepared for microscopy using glutaraldehyde buffered with PHEM and adjusted to be isotonic with seawater using sucrose, as described in Montanaro et al. (2016) (2.5% glutaraldehyde, 1.5X PHEM and 9% sucrose).

1.3 | Challenges With Single-Cell Scanning Electron Microscopy (SEM)

SEM is a high-resolution microscopy technique used to study the external morphology of an organism or tissue sample. Similar to TEM, sample preparation for SEM relies on the fixation and dehydration of specimens using glutaraldehyde, osmium tetroxide, and a graded ethanol series (as a solvent for dehydration) (Dykstra and Reuss 2003). Consequently, SEM with microscopic organisms also suffers from the risk of losing cells during liquid exchange steps. However, sample preparation for SEM is generally less time-consuming and resource-intensive than for TEM, as samples do not require resin embedding or ultrathin sectioning for data generation. SEM is a more accessible technique for ultrastructural studies of uncultivated microbial eukaryotes, as reflected in the vast number of published descriptions of uncultivated taxa that incorporate SEM imagery. Different strategies have been employed to obtain SEM data for single cells, such as using poly-L-lysine coated coverslips as a surface for cells to adhere to, placing cells directly inside Swinex filter holders, or containers

enclosed using membrane filters that can hold and retain cells during liquid exchanges, similar to the approach we describe here (Chantangsi et al. 2008; Lax et al. 2019; Leander 2006; Truby 1997; Rueckert and Leander 2008). Despite the demonstrated success of these techniques in the literature, the methodological descriptions of how single cells were prepared for SEM in these studies often lack sufficient detail to be easily reproducible.

Here, we provide a reproducible and reliable approach to the preparation of single cells for TEM and SEM that can be applied to a wide range of uncultivated protists with a sample size as small as one individual cell. Our approach uses inexpensive materials that can be commonly found or easily acquired in most labs, while the risk of losing cells is minimized, and in the case of TEM, a single cell can be observed and followed through the entire preparation process. Single cells are flat-embedded, so the location and position of cells within polymerized resin blocks can be easily identified using an inverted light microscope. This ensures that preparing single cells for ultrathin sectioning is relatively straightforward. We aim to increase the accessibility of electron microscopy techniques on uncultivated protists by providing a well-documented protocol that can be performed both in the lab and in the field.

2 | Material and Methods

2.1 | Preparation of Single Cells for TEM

Our approach to preparing uncultivated protists for TEM requires building a small container, hereby referred to as the “TEM fixation plate”, that houses individually isolated cells. This apparatus facilitates single-cell fixation, dehydration, and resin infiltration while minimizing cell resuspension and loss during liquid exchanges (Figure 1A). This protocol has been published in protocols.io ([dx.doi.org/10.17504/protocols.io.x54v953xql3e/v1](https://doi.org/10.17504/protocols.io.x54v953xql3e/v1)). The concentrations of reagents and length of time we present for each of the following steps should be effective for the majority of protists. However, these conditions can be adjusted as needed based on what has been previously used in previous studies of organisms related to the organism of interest. We have included the conditions for time-dependent steps for each organism prepared using this method in Table S1.

2.1.1 | Construction of the TEM Fixation Plate

The TEM fixation plate comprises one or two baskets attached to the base of a 60 mm polypropylene Petri dish (Figure 1A). Cells will be contained within the baskets for the majority of the sample preparation process. Prepare baskets from shortened pipette tips by removing the tapered ends of 1000 μ L pipette tips using a single edged razor, leaving 1.5 cm at the blunt end of each pipette tip to form a basket (arrowheads; Figure 1A) (Video S1). Heat the ends of a pair of fine forceps (e.g., dissecting forceps, Electron Microscopy Sciences) until red-hot (e.g., by using an alcohol lamp), and use them to punch ~8–10 evenly spaced small holes in the sides of the baskets. Holes should be placed in two rows, with each row about

1–2 mm from the ends of the basket (arrows; Figure 1A). It is essential that the holes are large enough to facilitate liquid exchange during the washing steps, but small enough so that the adhesive nature of the liquid fixative keeps the fixative within the chamber while gathering single cells; generally, the diameter of the hole is about 0.7–0.8 mm. Practically, if the holes are too small, liquid will be unable to enter the chamber during the washing steps. However, if the holes are too large, the fixative will leave the chamber haphazardly through the holes prior to the washing steps. Therefore, a balance in the diameter of chamber holes must be obtained. Alternatively, a heated 22-gauge needle can also be used to punch 0.7–0.8 mm holes in each basket.

Using a waterproof silicone sealant (such as Silicone Ultra All Purpose Premium Waterproof Sealant Aquarium Safe, DAP Canada), attach the prepared baskets to a polypropylene Petri dish (arrowheads; Figure 1A). Use a minimal amount of silicone so that only the edges of the basket contain silicone while ensuring that the silicone evenly coats the basket edge. The quantity of baskets within a single Petri dish can vary; however, the fixation timing must be consistent across every basket within a single TEM fixation plate, because they will all experience liquid exchange simultaneously during washing steps. Once single cells are ready to be embedded in an epoxy resin, the baskets will be removed from the fixation plates, leaving cells behind; therefore, it is important to keep the organism type consistent within one plate. In other words, only use one plate to fix multiple cells of one species rather than numerous different species, unless they can be easily distinguished. 15 mL Falcon tube lids are attached to the base of the plate using silicone sealant to reduce the available volume of each plate, so that smaller volumes of solutions and liquid waste are required (double arrowheads; Figure 1A). Allow the plates to sit exposed to air for 12–24 h (or however long is specified by the manufacturer) to allow the silicone sealant to cure. Additionally, poly-L-lysine can be applied to the bottom of the chambers to increase the ability of cells to stay in place during the process. This is particularly useful with smaller cells (< 20 μ m).

We provide manufacturer information for the exact materials used to construct the TEM fixation plates in Table S2, however, the materials used to construct the TEM fixation plate are not prescriptive. Construction of these plates can be accomplished using similar alternative supplies, if necessary, based on the availability of the materials.

2.1.2 | Aldehyde Fixation for TEM

Steps 1–3 should be performed inside a fume hood or in a well-ventilated area.

1. Fill the basket to about three-quarters full with your fixative solution of choice. We recommend using a fixative containing 2.5% glutaraldehyde buffered with PHEM (Montanaro et al. 2016). Sucrose can be used to adjust the osmolarity of the fixative solution to match the native osmolarity of the specimen. Place individual cells directly into the basket containing the fixative using a hand-drawn

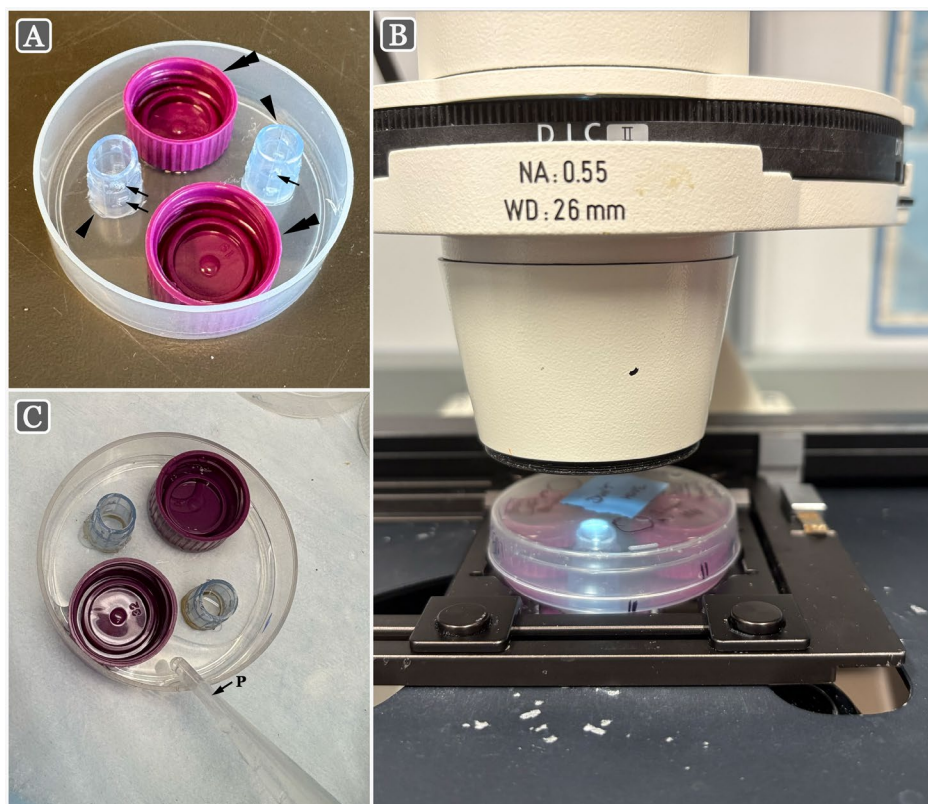


FIGURE 1 | Preparation of single cells for transmission electron microscopy (TEM) using TEM fixation plates. (A) General structure of the TEM plates used for single-cell preparation. Baskets constructed from 1000 µL pipette tips (arrowheads) are affixed to the base of a polypropylene Petri dish using waterproof silicone sealant. Holes made in baskets that facilitate liquid exchange are shown (arrows). Lids from 15 mL Falcon tubes (double arrowheads) are placed inside the dish to reduce its internal volume, allowing smaller volumes of waste associated with fixatives and buffers used during processing. (B) Fixatives and buffers can be added and removed from TEM plates containing single cells using a plastic transfer pipette (P). Liquids should be added and removed from outside of each basket to avoid disturbing or resuspending cells. (C) Cells can be visualized at any point during the sample preparation process using an inverted light microscope, except during steps when a fume hood is recommended.

micropipette or any other single-cell isolation device. Allow the cells to settle to the bottom of the basket before moving the plate. Single cells can be viewed inside the basket prior to and during fixation using an inverted light microscope (Figure 1B). Cover the plates to avoid exposure to fixatives and buffers while viewing samples.

2. Allow fixation to occur for 20–90 min at the temperature of the environment from which the cell(s) of interest were isolated. This length of fixation should be suitable for most single cells, but it can be increased for larger organisms (Table S1). The following steps all rely on the exchange of liquids inside the baskets containing cells (Figure 1C) (Video S1). To minimize cell loss and avoid resuspending single cells, all exchanges must be performed by adding and removing liquids outside of the baskets using a transfer pipette (P; Figure 1C). The holes in the sides of each basket will allow liquids to transfer between the baskets and the rest of the plate. It is crucial to perform liquid exchanges slowly to avoid resuspending cells. Allow the plates to sit for 5 min after the addition of each reagent (particularly when incubating cells in ethanol and acetone) to allow resuspended cells to settle to the bottom of the basket before removing liquid. This length of time also ensures that sufficient liquid exchange has occurred.

3. Wash cells three times using your preferred wash solution for 5 min each, as described above (Figure 1C). Wash solutions can contain the buffers used during fixation (Montanaro et al. 2016), but distilled water or filtered sea-water can also be used (depending on the native environment of the fixed organism).

Optional stopping point: Samples can be stored in the wash solution for up to 10 days at 4°C–8°C.

2.1.3 | Osmium Tetroxide Post-Fixation and Staining for TEM

Steps 4–6 must be performed inside of a fume hood.

4. Remove the wash solution from the plate (as described above) so that only the baskets remain partially filled with wash solution. The liquid levels inside each basket should drop until just below the lowest hole in the basket. Here, it is important that no moisture or leftover wash solution remains in the TEM plate outside of the baskets.
5. Add 4% osmium tetroxide into the basket using a plastic transfer pipette so that the final concentration of osmium tetroxide, when mixed with the remaining wash solution

within the basket, is 1%. Place a lid on each plate and allow post-fixation to occur for 1–2 h in the dark at ambient temperature. Both the concentration of osmium tetroxide and the post-fixation time can be increased to result in greater contrast of specimens when producing images. If greater contrast is desired, a 1.5 to 2 h incubation in 2% osmium tetroxide can be performed.

Once samples have been post-fixed with osmium tetroxide, perform a series of washes as described above. After 3–4 washes to ensure complete removal of osmium tetroxide, baskets can be examined with an inverted microscope to confirm that cells have not been lost at this stage (Figure 1B). Avoid moving the plates once the ethanol washes have begun, as it is especially easy to disturb cells contained in ethanol or acetone.

6. Wash cells three times for 5 min each using the wash solution that was used in Step 3.
7. Wash cells three times for 5 min each with distilled water.
8. Dehydrate samples using a graded ethanol series by incubating cells in ethanol solutions of increasing concentrations (using the same liquid exchange technique implemented during washes). We recommend using the following graded ethanol series: 30%, 50%, 70%, 85%, 90%, 95%.
9. Incubate cells in 100% EtOH three times for 5 min each. It is essential to remove as much ethanol as possible during each step.
10. Incubate cells in a 1:1 acetone ethanol mixture for 5 min.
11. Incubate cells in acetone twice for 10 min each. Cover the plate during this step to minimize acetone evaporation.

2.1.4 | Resin Infiltration and Embedding for TEM

Embedding biological specimens within an epoxy resin relies on using a series of resin-acetone mixtures to facilitate resin infiltration into organic material without distorting the tissue. Typically, resin infiltration steps start at 25% resin-acetone and are incrementally increased until samples are contained in 100% resin.

Because most protists are microscopic and relatively easy to infiltrate with resin due to their small size, we typically start with a 50% resin-acetone (Spurr's Resin, Electron Microscopy Sciences) mixture. The starting concentration for the acetone-resin mixture should be adjusted depending on how sensitive the tissue is to resin infiltration and on the resin of choice. Samples prepared using more viscous resins will require a lower resin-acetone ratio at the start of the resin infiltration steps. Due to the volatility of acetone, it will quickly evaporate from the resin mixture. With this in mind, we recommend leaving plates containing 50% resin-acetone partially uncovered and exposed to air to facilitate evaporation.

12. Infiltration in 50% resin-acetone must occur for a minimum of 1–2 h, depending on cell size; however, this step

can be extended to overnight. After 1–2 h, the acetone will have evaporated from the resin-acetone mixture, leaving the prepared cells in 75%–100% resin.

13. At this point, samples can be incubated in 100% resin immediately, because the evaporation process has simulated a resin-acetone gradient that prevents tissue distortion. 100% resin should be primarily added to the outside of the baskets in the same manner as wash solutions were added. Once the TEM fixation plate is full of resin, then a few drops of resin can be placed directly into the basket. This helps remove any remaining resin-acetone mixture inside the baskets that could influence polymerization.

Cells should be incubated in 100% resin three times before the final embedding step to ensure that cells are fully infiltrated and that all the remaining acetone was removed. Each incubation should be left for a minimum of 1 h for infiltration to occur, but it can be extended for up to 12–16 h. Incubation times should be increased to a minimum of 2–4 h for larger samples (> 100 µm).

Our TEM preparation method requires flat-embedding single cells (Figure 2A). This is achieved by removing all of the excess resin from the TEM preparation plate, except the resin contained within the basket. Then, the baskets can be gently and slowly removed from the base of the Petri dish using forceps. The single cells should remain at the base of the Petri dish contained in resin. However, if there was too much silicone applied to the baskets during the initial TEM plate construction, some cells can be lost when the baskets are removed.

14. Remove the baskets from each plate as described above. Next, add a small amount of pure resin to form a thin, even layer covering the entire bottom surface of the plate. Cover the Petri dish and place in an incubator at the temperature and time specified by the manufacturer's instructions for polymerization to occur.

2.1.5 | Finding and Mounting Your Cells of Interest for TEM Sectioning

Our approach to finding and mounting cells of interest is adapted from Reymond and Pickett-Heaps (1983). Polymerized resin disks can be easily removed from the Petri dish which they were polymerized in and directly viewed using an inverted light microscope (Figure 2B). Candidate cells for further processing for TEM can be identified. We recommend marking the surface of the resin using a fine-tip permanent marker by drawing a circle around the cells of interest. The piece of resin containing cells for TEM can be removed from the resin disk using a Dremel tool (Figure 2C). Cells can be visualized at high magnifications using an inverted light microscope, and the position of individual cells within the resin can be observed (arrowhead; Figure 2D). The surface of the resin can be marked with a series of guiding lines to indicate which edge of the resin block should be mounted on an empty resin block so that the cell is in the perfect position for sectioning (arrowhead; Figure 2E). For example, suppose you wish to obtain cross sections from the anterior end of a particular cell. In that case, a guiding line should be drawn near the posterior

end of the cell. A Dremel tool can be used to smooth the edge of the resin block, allowing it to be mounted flush against an empty resin block and aligned precisely with the guiding line to ensure the cell is correctly oriented for trimming, facing, and sectioning (Figure 2F–G).

2.1.6 | Preparation of *Dinema* sp. (Euglenida) for TEM

The transmission electron micrographs presented in Figure 3 represent an uncultivated marine euglenid, *Dinema* sp. Cells were isolated from intertidal samples collected at Manson's Landing Lagoon, Cortes Island, BC, Canada (50.069559, -124.979938). Two individually isolated cells of *Dinema* sp. were prepared using our TEM preparation protocol. Cells were fixed using 2.5% glutaraldehyde (Electron Microscopy Sciences) buffered in 1.5X PHEM (prepared as per Montanaro et al. 2016) and 9% sucrose. Washing steps 3 and 6 were performed using filtered sea water. Osmium tetroxide (Electron Microscopy Sciences) fixation occurred for 90 min using 2% osmium tetroxide for increased contrast. One cell (pictured in Figure 3) was micrographed throughout the preparation process. The *Dinema* sp. cells were embedded in Spurr's Resin (Electron Microscopy Sciences), and the cell of interest was

mounted for ultramicrotomy as described above. Ultrathin sections (70 nm) in cross section were obtained from the anterior region of the cell and were mounted on formvar-coated slot grids. Grids containing ultrathin sections were not stained prior to microscopy. Micrographs were obtained using a Tecnai Spirit Transmission Electron Microscope at the Bioimaging Facility (University of British Columbia).

2.2 | Preparation of Single Cells for SEM

Our approach for single-cell SEM preparation involves building a small container, hereby referred to as the “SEM baskets”, designed to house single cells during the fixation, dehydration, and critical point drying, while minimizing cell resuspension and loss during liquid exchanges (Figure 4A). This protocol has been published in protocols.io ([dx.doi.org/10.17504/protocols.io.rm7vz9pp2gx1/v1](https://doi.org/10.17504/protocols.io.rm7vz9pp2gx1/v1)).

2.2.1 | Construction of SEM Baskets

Prepare baskets to contain cells by removing the tapered ends of 1000 μ L pipette tips, leaving 1.5 cm at the blunt end of each

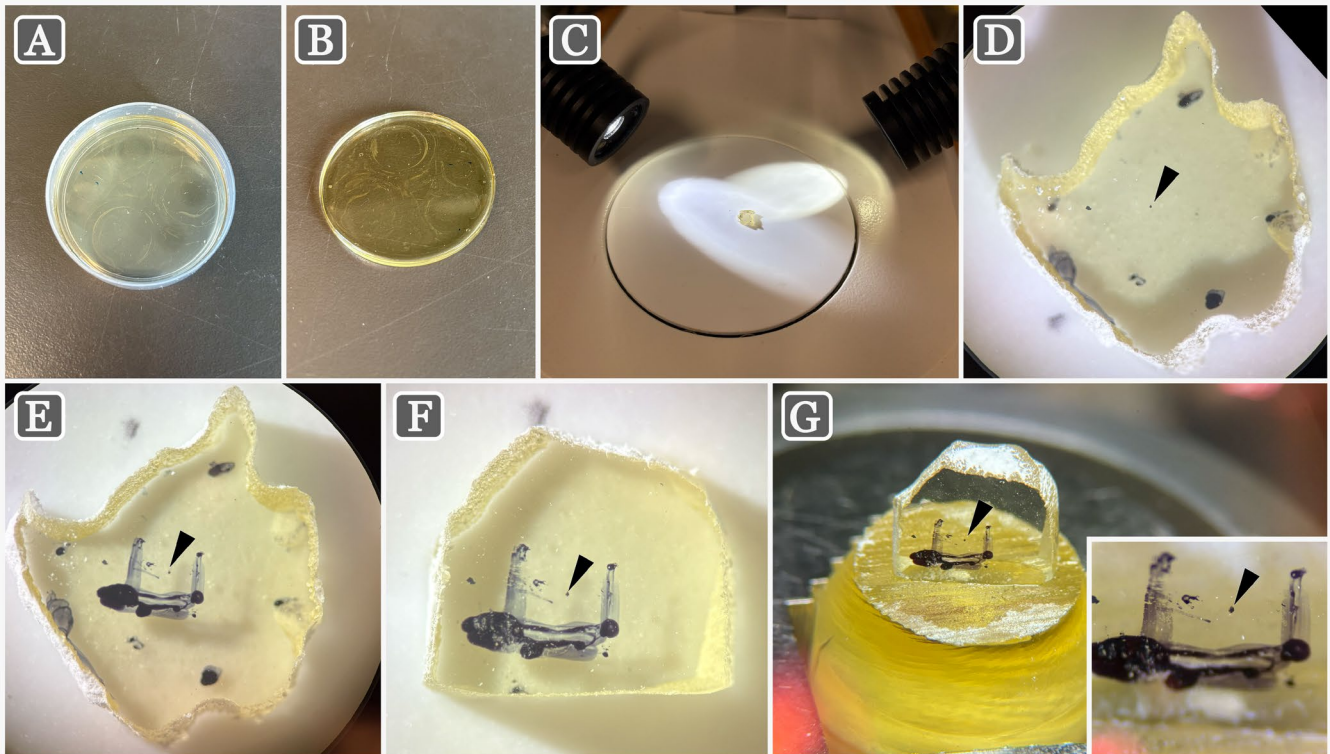


FIGURE 2 | Preparing flat-embedded single cells for ultramicrotomy. (A and B) Cells are flat embedded within the Petri dish used for fixation and dehydration upon the removal of baskets and falcon tube lids. The resulting polymerized resin puck can be scanned for fixed cells using an inverted light microscope or stereoscope. The position of cells within the resin is marked using a fine-tip marker. (C–E) The resin containing the cell of interest can be excised from the larger resin block using a Dremel tool. Guiding lines are then drawn around the embedded cell to aid in precise orientation. Two lines are marked parallel to the cell's *x*-*y* axis, and a third line is drawn perpendicular to the cell. (F) The edges of the resin block are polished using a Dremel tool, following the guiding lines drawn around the cell of interest. The base of the block (the surface to be adhered to the empty resin block) is trimmed to be parallel to the lower horizontal guiding line to ensure proper orientation for ultramicrotomy. (G) The prepared resin block containing the cell of interest is mounted on an empty resin block and can be observed using an ultramicrotome. Inset: High magnification view of the cell contained within the resin block, ready to be trimmed and faced for ultramicrotome sectioning.

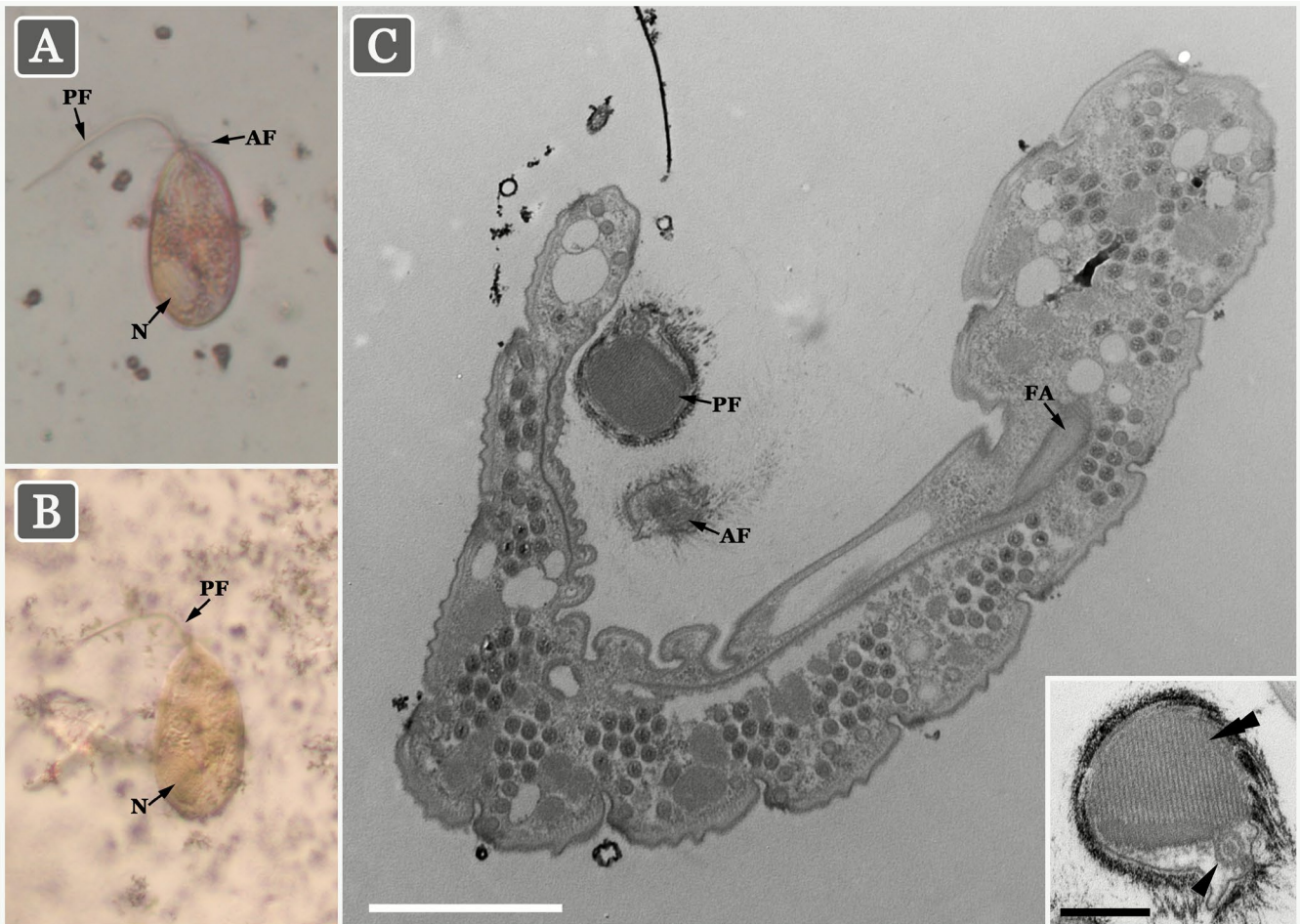


FIGURE 3 | Light (LM) and transmission electron micrographs (TEM) of *Dinema* sp. as observed through the fixation, dehydration, and embedding process for single cell TEM preparation. (A) A single *Dinema* sp. cell contained within a TEM plate basket after post-fixation using osmium tetroxide, visualized during an ethanol (30%) incubation step. The posterior flagellum (PF) and anterior flagellum (AF) remained intact during the fixation process. The nucleus (N) of the cell is also shown. (B) *Dinema* sp. embedded in Spurr's epoxy resin prior to ultramicrotome sectioning, showing the posterior flagellum (PF) and nucleus (N). (C) Low magnification TEM through the vestibulum of the same *Dinema* sp. cell as shown in panels (A and B), in cross section. Posterior flagellum (PF), anterior flagellum (AF), and the emergence of the feeding apparatus (FA) are shown. Scale bar = 2 μ m. Inset. High magnification TEM showing fine detail of the paraxial rod (double arrowhead) and axoneme (arrowhead) of the posterior flagellum. Scale bar = 500 nm.

pipette tip (arrowhead; Figure 4A). Coat the edge of the pipette tip end in waterproof silicone sealant and attach it to an Isopore membrane filter (Millipore Sigma) with an appropriate pore size for the organism of interest (double arrowheads; Figure 4A). We recommend using membranes with a pore size of $> 2 \mu$ m to ensure proper liquid exchange occurs during washing steps. Use a minimal amount of silicone so that only the edges of the basket contain silicone while ensuring that the silicone evenly coats the basket edge, creating a seal between the basket and the membrane. Allow the baskets to sit exposed to air for 12–24 h (or however long specified by the manufacturer) to allow the silicone sealant to cure.

We provide manufacturer information for the exact materials used to construct the SEM baskets in Table S2; however, the materials used to construct the SEM baskets are not prescriptive. Construction of these baskets can be accomplished using similar alternative supplies, if necessary, based on the availability of the materials.

2.2.2 | Aldehyde and Osmium Tetroxide Fixation for SEM

Place SEM baskets inside a 24-well plate (Figure 4B). Process only 4–6 SEM baskets at a time to leave the majority of the wells in the plate available for the washing steps.

1. Fill each basket approximately halfway with the fixative solution (typically a buffer like PHEM containing 2.5% glutaraldehyde). Add the fixative solution to the well outside of the basket until it is about halfway up the side of the basket to prevent the liquid level from dropping inside of the basket. Place individual cells for SEM directly inside of the baskets.
2. Allow fixation to occur for 20–90 min at the temperature of the environment from which the cells were isolated. Steps 3–7 require washes and liquid exchanges as shown in Figure 4C and Video S2. These are performed by removing the basket from the well and placing it on a clean

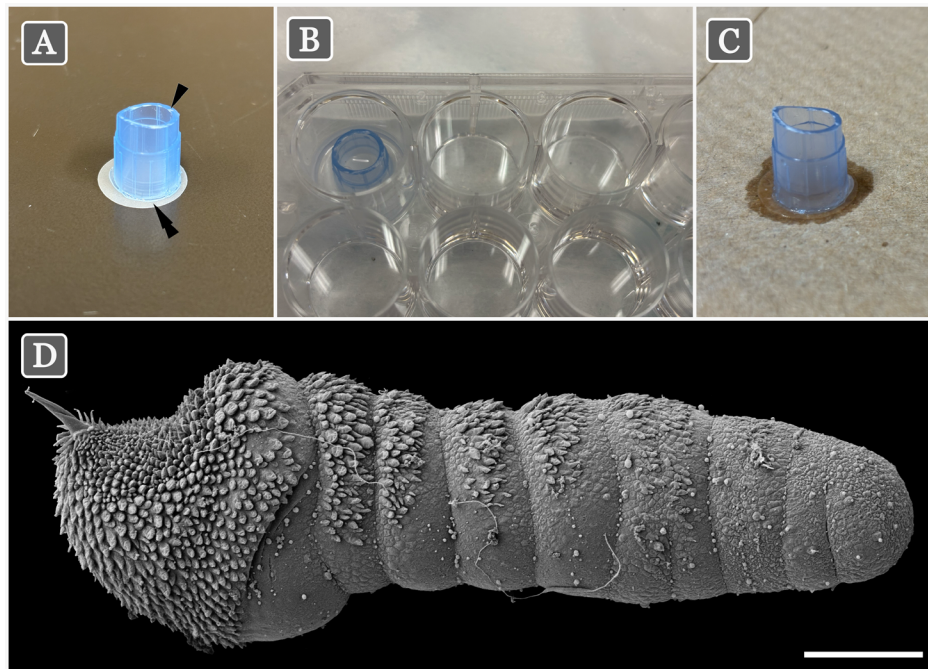


FIGURE 4 | Preparation of single cells for scanning electron microscopy (SEM) using custom-made baskets. (A) SEM preparation basket constructed from the blunt end of a 1000 µL pipette attached to a 5 µm isopore membrane filter using silicone. (B) An SEM basket containing fixed cells inside of a 24-well plate during a washing step. The basket and the well are both filled ~50% of the way with filtered sea water. (C) SEM baskets containing fixed cells are placed on a piece of paper towel to facilitate liquid exchange. Fresh reagent (depending on the step of SEM processing) is placed directly inside of the basket as it is drained to ensure cells are never exposed to air. (D) A single parasite of *Haplozoon* sp. (Dinoflagellata) prepared using this approach for SEM. Scale bar = 10 µm.

paper towel. The paper towel will draw liquid through the membrane, reducing the fluid level inside the basket. As liquid drains out of the basket, use a transfer pipette to add the new liquid/solution to the basket until a full exchange occurs. Then place the basket into a new well containing the next solution (e.g., wash solution, distilled water, or ethanol) and incubate for 5 min for every wash to ensure adequate liquid exchange.

3. Following fixation, wash cells contained within baskets three times using a wash solution (e.g., distilled water, filtered seawater, or a buffer).
4. This step must be performed inside of a fume hood. Fill the baskets (and the well containing each basket) approximately halfway with 1% osmium tetroxide for post-fixation. Allow cells to fix for ~10–20 min at room temperature.
5. Wash the cells within baskets 3 times in a wash solution (e.g., distilled water, filtered seawater, or a buffer).
6. Wash cells 3 times in distilled water.
7. Dehydrate cells using a graded ethanol series (30%, 50%, 70%, 85%, 90%, 95%) using the same technique that was used to wash the cells following fixation.

Optional stopping point: Fixed cells can be stored long term in 70% ethanol if required

8. Wash cells in 100% ethanol 3 times prior to critical point drying to ensure adequate dehydration of samples. Baskets

can be placed directly into a critical point dryer. Dried membranes can then be mounted on aluminum stubs using adhesive tabs, and the pipet tip part of the baskets can be removed before sputter coating.

2.2.3 | Preparation of *Haplozoon* sp. (Dinoflagellata) for SEM

The scanning electron micrograph presented in Figure 4 shows an uncultivated marine dinoflagellate, *Haplozoon* sp. A single host specimen was collected during a SCUBA dive in Hyacinthe Bay, Quadra Island, BC, Canada (50.114722, –125.224722) in May 2024. Two haplozoan cells were isolated, rinsed in filtered seawater, and prepared for SEM using the protocol provided in this study. Briefly, the cells were fixed with 2.5% glutaraldehyde in filtered seawater in a basket placed in a 24-well plate in the field. The plate was sealed with Parafilm to prevent liquid evaporation. The plate was then transported to the University of British Columbia for further processing. Washing steps 3 and 6 were performed using filtered seawater. Osmium tetroxide fixation was carried out for 10 min using 1% osmium tetroxide. Ethanol dehydration was performed in a graded series, starting with 30%, followed by 50%, 70%, 80%, 85%, 90%, 95%, and then 100% ethanol three times. The basket was transferred to a larger container submerged in 100% ethanol for critical point drying using the Tousimis Autosamdri 815B Critical Point Dryer. Once dried, the entire basket was mounted onto an SEM stub using an adhesive tab. The plastic tip of the basket was removed, leaving only the membrane on the stub, which was then ready for

sputter coating. A 2 nm layer of platinum-gold was applied using either the Cressington 208HR High-Resolution Sputter Coater or the Leica EM ACE600 Coater. Images were acquired using the Zeiss Crossbeam XB350 at the UBC Bioimaging Facility.

Table S2 lists the manufacturer information and materials used in both the SEM and TEM approach described above. Videos S1 and S2 demonstrate how to construct TEM plates, SEM baskets and how to perform wash steps. This manuscript was minimally edited for syntax and grammar using ChatGPT and Grammarly.

3 | Results

To demonstrate the success of our TEM approach, we prepared a single cell belonging to the heterotrophic euglenid *Dinema* sp. (Figure 3). This isolated cell of *Dinema* sp. was tracked throughout the TEM preparation process beginning with fixation and dehydration (Figure 3A) and continuing through embedding in Spurr's epoxy resin (Figure 3B), using an inverted light microscope, ultimately allowing us to obtain high-resolution TEM images of the cell (Figure 3C). The nucleus and posterior flagellum of *Dinema* sp. could be clearly observed in both the fixed stage of sample preparation (Figure 3A) and even once the cell was embedded in Spurr's resin (Figure 3B). Because the anterior and posterior regions of the cell remained clearly distinguishable under light microscopy after embedding in polymerized resin, the cell could be easily positioned for ultramicrotomy, allowing us to obtain TEM images in cross section through the anterior region of the cell at the level of the vestibular opening (Figure 3C).

The posterior flagellum (PF) and anterior flagellum (AF) were situated within the vestibular opening, running in parallel along a groove on the ventral side of the cell. Fine ultrastructural detail of microtubular axonemes (arrowhead) and the paraxial rod (double arrowhead) of the posterior flagellum are shown (inset; Figure 3C). The lattice-like structure of the paraxial rod and individual microtubules that compose the axoneme was easily observed despite the fact that grid staining using uranyl acetate and lead citrate was not performed.

To demonstrate the success of our SEM approach, we prepared a single *Haplozoon* sp. cell isolated from a specimen of the bamboo worm, *Axiiothella* sp. (collected at Quadra Island, BC, for SEM) (Figure 4D). The cell was ~70 µm in size and was free of any debris.

4 | Discussion

There have been previous attempts to overcome the challenges of preparing single cells for electron microscopy, particularly for TEM and for studies interested in the ultrastructure of single cells that represent a life stage of multicellular organisms (e.g., oocytes) (Truby 1997; He et al. 2006). Many of the problems associated with preparing single reproductive cells of multicellular organisms parallel those that occur during the preparation of uncultivated protists. For example, loss of cells can occur during liquid exchanges, and it can be challenging

to identify cells in a polymerized resin block before ultramicrotome sectioning (He et al. 2006). Approaches to overcome these challenges have included embedding cells in agarose to prevent cell loss (Faure et al. 1992; He et al. 2006; Wakeman et al. 2014) or using willow leaf triangles to mark the location of specimens for sectioning (He et al. 2006). In the context of protists, efforts have been similarly aimed at improving single-cell preparation for TEM. Truby (1997) describes a method for preparing single dinoflagellate cells for TEM that involves embedding cells using agarose inside BEEM capsules after fixation, where they are subsequently processed and embedded in resin. While this technique effectively prevents cell loss during the fixation, dehydration and resin infiltration process, the orientation of cells for ultrathin sectioning depends on how the cells naturally settle in the agarose. In other words, it is impossible to obtain precise cross sections or longitudinal sections through particular regions of a single cell using this technique if they do not embed in the appropriate orientation; a challenge that is overcome by flat embedding.

Unfortunately, few of these techniques are described in detail, and each has limitations. For instance, while techniques that require embedding cells in agarose avoid cell loss, these samples may not be suitable for flat-embedding and can lead to issues with resin polymerization if any remaining ethanol or acetone is trapped within the sample (Mollenhauer 1993). This is an important distinction between our technique and any other published approaches. Using our TEM preparation method, where cells are contained within baskets and then flat-embedded within the plate, we can: (1) prevent cell loss throughout the preparation process; (2) ensure cells can be easily identified within resin blocks for sectioning; and (3) flat-embed cells in an epoxy resin so they can be positioned in any orientation for sectioning. Our single-cell TEM preparation technique successfully enabled the acquisition of precise cross-sectional TEM images through a specific region of interest of a single cell (*Dinema* sp.). We were able to obtain high-resolution light micrographs of the flagella and nucleus in a fixed cell, as well as corresponding TEM images for the same individual cell.

While other SEM studies of uncultivated protists use various techniques to prepare cells while minimizing cell loss, the methodology sections of these studies are usually not written in sufficient detail to be easily reproducible. We provide a detailed description of an approach similar to approaches previously used (Chantangsi et al. 2008) that can be easily applied to a wide range of uncultivated taxa and with small sample sizes. Obtaining SEM of uncultivated *Haplozoon* sp. cells was straightforward using our SEM approach; cell loss was minimized during the fixation and dehydration process, and cells were debris-free. Haplozoans are an interesting group of parasitic dinoflagellates that infect bamboo worms. However, both the hosts and the parasites are rarely encountered (though this can vary by location and season), making them challenging to study (Park et al. 2023). Using the protocols described in this study, even a few cells or a single cell can be imaged to obtain detailed surface ultrastructural information.

Our approach for preparing uncultivated protists for electron microscopy is practical, reliable, and can be easily implemented

in the field. In addition to the micrographs presented in this manuscript, the success of both our SEM and TEM approach has been recently demonstrated through their implementation (sometimes with modifications) for the ultrastructural descriptions of several different protists published by authors of this study, including Wakeman and Horiguchi (2018), Wakeman, Yabuki, et al. (2018), Yamaguchi et al. (2018), Yamamoto et al. (2020), Wakeman (2020), Wakeman, Yamaguchi, and Horiguchi (2018), Wakeman et al. (2021), Iritani et al. (2021), Yokouchi et al. (2022), Van Steenkiste et al. (2023), Yamaguchi et al. (2023), Odle et al. (2024), Palka et al. (2025), and Martinez et al. (2025). However, as far as we are aware, this is the first detailed description of this approach in the form of a step-by-step protocol that can be easily adapted for ultrastructural studies of other uncultivated protist lineages.

Author Contributions

M.V.P., K.C.W., and B.S.L. conceived and guided the study. M.V.P. and G.L. prepared specimens and acquired TEM images. E.P. prepared specimens and acquired SEM images. M.V.P. obtained the images depicting the approach and generated the supplementary videos. K.C.W. invented the materials and methods used in the described approach. M.V.P. built the figures and wrote the first draft of the manuscript. M.V.P., E.P., K.C.W., G.L., and B.S.L. edited subsequent iterations of the manuscript. B.S.L. and K.C.W. funded the research.

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Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Sample preparation conditions used to prepare published transmission electron micrographs of various protists using our protocol. **Table S2:** Manufacturer information for the materials used to produce micrographs of *Dinema* sp. and *Haplozoon* sp. using our single-cell EM protocols. **Video S1:** Demonstration of how to construct a TEM plate and how to perform washing steps/liquid exchange steps. **Video S2:** Demonstration of how to construct SEM baskets and how to perform washing/liquid exchange steps.