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Refining cryptophyte identification: matching cell fixation methods to FISH hybridisation of cryptomonads

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Abstract The Division Cryptophyta, Class Cryptophyceae, contains ecologically important microalgae that are found in all aquatic habitats. The identification of the Cryptophyta is challenged by a need to examine species in the scanning electron microscope or transmission electron microscope to visualise features needed to identify its species. Molecular verification is becoming increasingly important for this group because of its polymorphic haploid and diploid cells of the same species with different morphologies. Thus, for routine monitoring programmes, this group is not usually identified beyond the level of class and that is done only if the samples are routinely examined with a fluorescent microscope or with flow cytometry, and the cryptophytes are counted based on the natural orange fluorescent of their phycobilin pigments. In order to use rRNA probes, the cells must be fixed for permeabilisation of the cell membrane for probe penetration. Here, we present a test of routine fixation methods to determine the fixation that is most compatible for use in fluorescent in situ hybridisation methods with fluorescent microscopy and flow cytometer to facilitate cryptomonad identification.

Keywords Cryptophytes · Fixation methods · Flow cytometry · FISH hybridisation

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Introduction

The Cryptophyta are biflagellate unicellular microalgae, with a haploid–diploid life cycle. The microalgae are highly diverse and have a unique morphology and suite of pigments (Hoef-Emden et al. 2002, 2003). Their two classes are represented by plastid-containing (including three colourless *Cryptomonas* lineages) and aplastidic forms (genus *Goniomonas*). However, the identification of cryptophyte microalgae is a challenging task because certain preservatives, such as glutaraldehyde, rupture the cells, and species must be examined in the scanning electron microscope or transmission electron microscope with cryofixation to visualise features identifying the species (Clay et al. 1999). Few ecological studies are performed on cryptomonads because of these limitations (Cerino and Zingone 2006). Thus, for routine monitoring programmes, this group usually is not identified beyond the level of class and that is done only if the samples are routinely examined with a fluorescent microscope, and the cryptophytes are counted based on the natural orange fluorescence of their phycobilin pigments. They are often counted in flow cytometry based on their orange fluorescence (Li and Dickie 2001). Metfies et al. (2007) presented a set of molecular probes, based on ribosomal RNA genes, developed for the identification of Cryptophyceae at the class and clade or family level, which were tested for the specificity and for their applicability on a DNA microarray (Metfies and Medlin 2007), thus eliminating the problems associated with cell fixation and visualisation with electron microscopy. All of clade level probes were highly specific for the clades for which they were designed with the exception of clades 4–6, which recognises species in two clades: IV and VI of Marin et al. (1998). These same probes have been used with archived Lugol fixed samples and

Table 1 Summary of strains and probes used in this study

Tested organisms	Strain source	Probe	Clade according to Marin et al. (1998)	Clade according to Hoef-Emden et al. (2002)	Oligonucleotide sequence (5′–3′)
All species listed below		EUK 1209	n/a	n/a	GGGCATCACAGACCTG
		NON-EUK			GGAGGGCAAGTCTGGT
Cryptomonas pyrenoidifera Geit. (= *Campylomonas reflexa*)	CCMP 1177	CryptoB	n/a	n/a	ACGGCCCCAACTGTCCCT
Cryptomonas (= *Rhodomonas*) *baltica* (Karsten) Butcher	SAG 18.80				
Rhinomonas reticulata (Lucas) Novarino	PLY 358				
Pyrenomonas helgolandii Santore	SAG 28.87				
Cryptomonas irregularis Butcher	CCAP 979/7				
Rhodomonas sp.	CCMP 768				
Rhodomonas (= *Chroomonas*) *salina* (Wislouch) Hill et Wetherbee	PLY 544				
Hanusia phi Deane	CCMP 325				
Guillardia theta Hill et Wetherbee	CCMP 327				
Chroomonas sp.	CCMP 270				
Proteomonas sulcata Hill et Wetherbee	CCMP 1175				
Cryptomonas (= *Rhodomonas*) *baltica* Karsten		Crypt01-3	I	CRYP	TCATTACCCCAGTCCCAT
Cryptomonas curvata (= *Campylomonas reflexa*)					
Cryptomonas irregularis					
Rhinomonas reticulata		Crypt02	II	RHO	GTCCCACTACCCTACAGT
Pyrenomonas helgolandii		Crypt02-25			CATTACCCCAGTCCCATAACCAACG
Rhodomonas sp.					
Rhodomonas salina					
Hanusi phi		Crypt03	III	PROT	TTCCCGCGCACCACGGTT
Guillardia theta					
Chroomonas sp.		Crypt4-6	VI	CHRO	CAAGGTCGGCTTTGAATC
Proteomonas sulcata		Crypt5-3	V	PROT	GTCCCAACGCCCCTCAGT

counted with a solid-phase cytometer, the CHEMSCAN (Medlin and Schmidt 2010). Because a microarray (phylochip) is presently only semiquantitative (Metfies et al. 2010), as part of an evaluation of detection methods for cryptomonads, we have evaluated several methods for their use in distinguishing cryptomonads for more quantitative assessments. The method presented here is fluorescent in situ hybridisation (FISH) where cell format is retained. FISH hybridisation methods and probe specificity were already tested and optimised (Metfies et al. 2007), and the main goal of this paper was to test existing fixation methods to see which one provided the most compatible method with our optimised FISH protocols (Töbe et al. 2006).

Material and methods

Cultures of cryptomonads representing each of the molecular clades (Table 1) in Marin et al. (1998) for which molecular probes was developed (Metfies and Medlin 2007) were grown in IMR media (Eppley et al. 1967) or DY-IV medium (Andersen et al. 1997). Two to 5 mL of each culture were removed and fixed as described below.

Fixation and FISH on filters

Various fixation methods (Table 2) were tested to determine if existing cell preservation methods would be compatible with our existing optimised FISH protocols. Acid Lugol's preservative was prepared according to Throndsen (1978), and after 1 h of fixation, cells were then hybridised according to Töbe et al. (2006). Because of previous experience with Lugol fixed phytoplankton samples, we knew that we had to use an additional fixation method to ensure that the cell membrane was permeabilised for probe entry (Töbe et al. 2001). Following fixation of species with Lugol's iodine, samples were treated a saline ethanol fixative (Miller and Scholin 1996, 2000). Samples were also fixed with saline ethanol alone for 1 h, for 4 h and for 2 days. Further cells were fixed with Lugol's iodine and then fixed with 4% paraformaldehyde (PFA; Töbe et al. 2001) for 1 h at 4°C. Cells were also fixed with 4% PFA alone and fixed for 1 h at 4°C. Cells, following the PFA fixation, were not treated with saline ethanol because previous work had shown this not to be effective in reducing the autofluorescence (Medlin and Schmidt 2010). We processed cells from the PFA alone treatment with 50% dimethylformamide (DMF) to extract the chlorophylls to reduce the autofluorescence (Groben and Medlin 2005). We did not try to reduce substantially the cell's autofluorescence with other chemicals or with exposure to UV light as in Takahashi et al. (2005).

Afterwards, the cells were filtered as described in Töbe et al. (2006) and hybridised with their respective clade specific fluorescein isothiocyanate (FITC)-labelled oligonucleotide probe or class level probe (CrytoB), together with a Cy5-labelled EUK 1209R (Giovannoni et al. 1988; Table 1). The latter probe recognises many eukaryotes and served as a positive control in the hybridisation experiments. One filter piece was hybridised with ANTI-EUK1209R (Deere et al. 1998), a reverse complement of probe 'EUK' (Amann et al. 1990), and was used as a control for unspecific binding, and one additional filter fragment was hybridised without probe as control for autofluorescence of the algal cells. Our existing FISH hybridisation protocols were further optimised with respect to formamide concentration (20%) and stringency of the washing buffers as cited in Töbe et al. (2006) to ensure the brightest probe fluorescence. All fluorescence was visually evaluated with regards to intensity. All photographs were made with a Nikon fluorescent microscope with the following filter sets: FITC (B-2A) Ex: 450-490/ and for CY5 (BA520) Ex 590-650.

FISH in suspension for flow cytometry

Based on the results with various fixation protocols, cultures fixed with 4% PFA for 1 h at 4°C were hybridised in

Table 2 Comparison of hybridisation results among various fixation protocols

Fixation method	Cell morphology and autofluorescence
Lugol's preservative alone	Intact cells, high autofluorescence
Lugol's preservative plus subsequent saline ethanol fixation 1 h, RT	Intact cells, high autofluorescence
Saline ethanol fixation 1 h, RT	>50% cells damaged or ruptured
Saline ethanol fixation 4 h, RT	>50% cells damaged or ruptured
Saline ethanol fixation 2 days, RT	>50% cells damaged or ruptured
4% PFA 1 h, 4°C	Intact cells
4% PFA 1 h, 4°C plus subsequent saline ethanol fixation 1 h, RT	Intact cells, some autofluorescence[a]
Lugol's preservative plus 4% PFA 1 h, 4°C	Intact cells, high autofluorescence
4% PFA 1 h, 4°C, plus subsequent treatment with 50% DMF, 1 h, 4°C	Intact cells, but no reduction in autofluorescence

[a] These results are taken from another study (Medlin and Schmidt 2010)

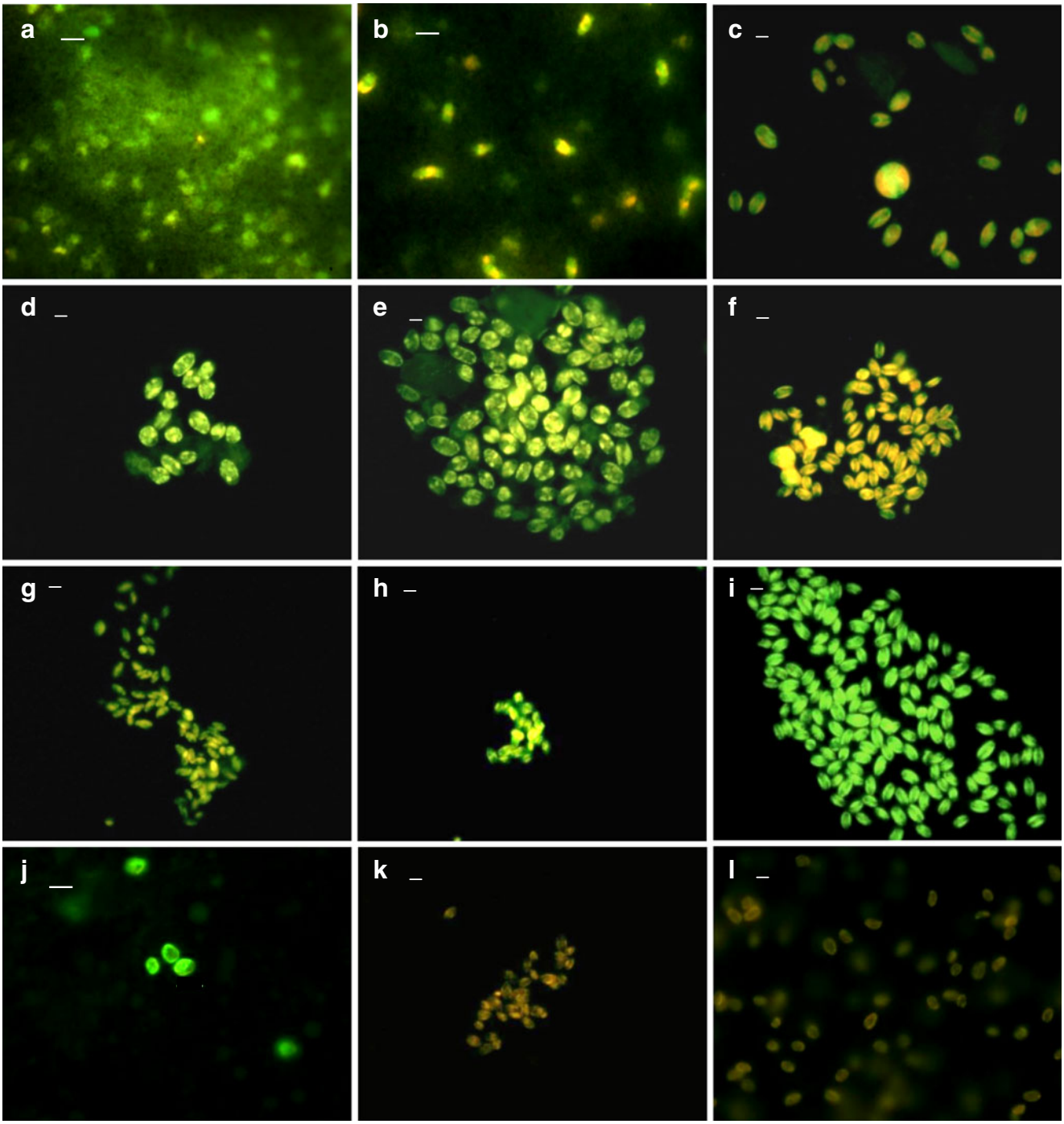

Fig. 1 Examples of different fixation procedures and specificity tests of rRNA probes detected by FISH with various cryptophycean algae. **a** *C. irregularis* hybridised with FITC-labelled probe CryptoB, fixation with saline ethanol. **b** *C. irregularis* hybridised with FITC-labelled probe CryptoB, Lugol's fixation. **c** *Rhinomonas reticulata* hybridised with FITC-labelled probe CryptoB, fixation with 4% PFA and DMF treatment. **d**–**l** Fixation with 4% PFA alone. **d** *Cryptomonas* (= *Rhodomonas*) *baltica* hybridised with FITC-labelled probe CryptoB. **e** *C. baltica* hybridised with FITC-labelled probe Crypt01-3. **f** *Pyrenomonas helgolandii*, hybridised with FITC-labelled probe Crypto02-25. **g** *Proteomonas sulcata* hybridised with FITC-labelled probe Crypto5-3. **h** *Rhodomonas salina* hybridised with FITC-labelled probe Crypto4-6. **i** *Pyrenomonas helgolandii* hybridised with FITC-labelled probe Crypt02. **j** *Hanusia phi* hybridised with FITC-labelled probe Crypt03. Negative controls showing autofluorescence: **k** *Rhodomonas salina* hybridised without a probe, **l** *Hanusia phi* hybridised without a probe. Magnification: **a**, **b**, **j** *scale bar*=10 μm, **c**–**l**, *scale bar*=10 μm

suspension with the class probe CryptoB according to Biegala et al. (2003) for flow cytometry purposes. Basically, the same steps as for FISH on a slide or filter are followed with the following exception. After spinning down the cells, the pelleted cells were treated for 1 h at 4°C with 50% DMF. Following fixation, the cells were passed through a FACSCalibur (Becton Dickinson, USA) flow cytometer to examine the fluorescent signal in analytical flow cytometry and intensities in red and orange fluorescence plotted.

Results and discussion

All of the cryptomonads showed strong autofluorescence after fixation with all of the methods (Figs. 1a–l). This autofluorescence ranged from yellow to yellow-green to bright orange, depending on the species and the size of their plastid. Thus, the anatomy of the cell dictated how much cytoplasm was available for localisation of the positive probe signal. All species tested reacted in the same way to each fixative. In no case was one species better fixed in one method than in another. Thus, we have illustrated each fixation method with a different species to show the range of species tested rather than to illustrate all methods with the same species. Saline ethanol fixation resulted in cell rupture (Fig. 1a) for all species. The majority of the cells were ruptured and this was true for all the species tested. Positive signals in intact cells could be seen, but there was lots of cell debris making the positive green probe signal rather diffuse, suggesting that free cytoplasm had been extruded onto the glass slide. Given the preferred use of saline ethanol over other more toxic preservatives by most scientists working with microalgae, especially toxic ones, means that more toxic protocols will have to be used for FISH with cryptomonads. This may preclude them being used under some shipboard conditions where fume cupboards are not available. In general, the fixation with Lugol's iodine resulted in the largest autofluorescence (compare the predominance of the yellow of the chlorophyll in the cells in Fig. 1b to all others). Cells also had a slightly fuzzy outline suggesting that the fixative had caused discharge of some trichocysts. The poor performance of the Lugol's fixation even followed by PFA fixation was disappointing in view of the many archived Lugol's fixed samples stored by various oceanographic institutes (see fixation of Lugol's archived material in Töbe et al. (2001) for bacteria and Medlin and Schmidt (2010) for cryptomonads). However, if one becomes used to the strong autofluorescence of the cells, it is easy to pick out the distinctive green fluorescence of the positive signal of the probe in the cell cytoplasm. Fixation was optimal with the toxic PFA (Figs. 1c–j). Subsequent treatment with saline ethanol was not performed here because we knew from previous experience that it did reduce but did not completely eliminate autofluorescence (see Medlin and Schmidt (2010) for field samples of cryptomonads processed this way). Further treatment with DMF to remove further the chlorophylls also did not reduce the autofluorescence (Fig. 1c). It may be that because the phycobilins are water-soluble pigments, other non-polar solutions should be investigated to try to remove these pigments from the cells to reduce their autofluorescence or expose the cells to UV light to bleach the chlorophylls (Takahashi et al. 2005). This was not tested here because the aim of our work was to test existing fixation methods with our standardised FISH protocol and not to optimise the FISH protocol for the cryptomonads because the FISH probes were specific (Metfies and Medlin 2007).

The Cy-5-labelled EUK probe worked in all cases as a counter stain to ensure that the hybridisation was successful (data not shown, see Medlin and Schmidt (2010) for a comparison of this probe to clade level probes). All FITC probes worked specifically in the FISH detection methods using 20% formamide and standard buffer concentrations for rinsing the cells (Töbe et al. 2006). FITC probes could be seen as bright green areas within the cells (Fig. 1c–e, g–j) or as a green outline surrounding a yellow cell (Fig. 1f). Bright green positive probe signals were sometimes restricted to small areas of the cytoplasm if the plastids were large and occupied most of the cell. Four different species were hybridised with the class level probe, CryptoB (Figs. 1a–d), to illustrate the compatibility of the fixation method with our standard FISH method. In addition, we have illustrated one example of each of the clade level

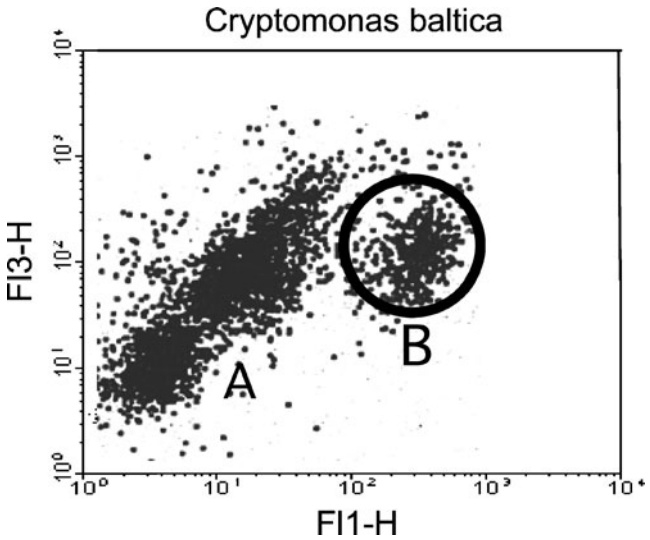

Fig. 2 Cytogram of *C. baltica* fixed by 4% PFA followed by DMF treatment hybridised with Crypto B. Cytoscan with probe is superimposed on the cytoscan without a probe. A shift in the fluorescent signal of cells without a probe (**a**) is seen when a probe is added to the cells (**b**). There is no overlap between the probe signal and the natural autofluorescence of the cells

probes tested (Fig. 1e–i). In one case, we tested a longer oligonucleotide probe for the same clade (clade 2 at 18 bp vs clade 2–25 at 25 bp; Figs. 1f, i). The longer probe gave a stronger signal as judged by brightness of the green positive signal, and this proved to be the case for each probe whose length was extended (see microarray results in Metfies et al. 2007, 2010). Each species is brightly labelled with no one species being better labelled than the other for all probes; the only difference being the size of the plastid, which appeared as a yellow or yellow-green body in the cell because of the strong autofluorescence of the plastid. A comparison of Figs. 1h, j with Figs. 1k, l provides a general indication of the amount of autofluorescence exhibited by each species as compared to a positive fluorescence of the FITC probe. *Cryptomonas baltica* has been referred to the genus *Rhodomonas* but in our study gave a positive signal with probe crypto01 and not crypto02, which suggests its taxonomic position should be re-examined or the identity of this culture rechecked. Also, if this culture is truly a *Cryptomonas* spp., then the fact that all *Cryptomonas* spp. are freshwater should be re-evaluated because this is a marine species. *Cryptomonas irregularis* also a marine species gave a weak signal with probe crypto01 (data not shown).

Given that the PFA produced the best fixation of the cells, this fixation method was used with FISH in suspension to analyse the cells with analytical flow cytometry. All probes were tested in the flow cytometer with one species from the respective clade, and a typical cytogram is shown in Fig. 2. A strong displacement of the signal with the probes vs cells with no probe could be shown, thus overcoming any autofluorescence exhibited by the cells. We include only one example to show the signal shift from of cells from a no probe position to a probe position in the flow cytometer because we considered that this was sufficient documentation that cells could be detected in the flow cytometer and that the intensity of the probe signal was high enough to shift it away from the no probe signal of the cell's autofluorescence. In some cases, the probe signal can be not strong enough to distinguish it from the cell's natural autofluorescence in flow cytometry (Lange et al. 1996). Because our study aimed to determine the optimal fixation method for both whole-cell fish hybridisation and flow cytometry, we did not address issues such as cell losses that are known to occur in FISH in both methods and the reader is referred to the extensive tests made by Biegala et al. (2003) to address the problem in FISH in suspension. Cell loss in FISH with fluorescent microscopy is known to occur but has never been quantitatively documented to our knowledge. A comparison of many molecular methods against traditional methods can be found in Godhe et al. (2007).

Because of the strong autofluorescence, which we did not diminish or reduce other than to try an exposure to DMF, a trained worker must be used to distinguish positive from negative cells (see positive signals in Figs. 1f) when examining cells with fluorescent microscopy. However, with the use of flow cytometry, the combination of the orange fluorescence with the green fluorescence of the probe will provide an enhanced signal to detect and identify cryptomonad cells down to the clade level. FISH hybridisation in suspension is easy to perform even by untrained personnel and then the addition of the mixture to the flow cytometer will provide a good discrimination of the cryptomonads to the clade level, which is better resolution of the diversity of this important coastal group of phytoplankton than is presently achieved. However, if FISH in suspension is to be used quantitatively, additional precautions must be addressed to prevent or minimise cell loss.

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