Chapter 2

FISH and Immunofluorescence Staining in *Chlamydomonas*

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Abstract

Here we describe how to use fluorescence in situ hybridization and immunofluorescence staining to determine the in situ distributions of specific mRNAs and proteins in *Chlamydomonas reinhardtii*. This unicellular eukaryotic green alga is a major model organism in cell biological research. *Chlamydomonas* is well suited for these approaches because one can determine the cytological location of fluorescence signals within a characteristic cellular anatomy relative to prominent cytological markers. Moreover, FISH and IF staining offer practical alternatives to techniques involving fluorescent proteins, which are difficult to express and detect in *Chlamydomonas*. The main goal of this review is to describe these powerful tools and to facilitate their routine use in *Chlamydomonas* research.

Key words: *Chlamydomonas reinhardtii*, Cell, FISH, Algae, Plant, Chloroplast, Flagella, Pattern formation, mRNA, Localization, Fluorescence microscopy, In situ hybridization, Confocal

1. Introduction

The unicellular eukaryotic alga *Chlamydomonas reinhardtii* is a model organism for research into a variety of cell biological processes, e.g., photosynthesis, chloroplast biogenesis, and flagella-based motility (1). Researchers benefit from a powerful set of methods, and the sequenced and annotated genomes of the chloroplast and nucleus (2, 3). Yet the tools involving fluorescence microscopy have been underexploited, in large part due to strong interference from chlorophyll autofluorescence and potent silencing of transgenes encoding GFP-tagged proteins (4–8).

Fluorescence in situ hybridization (FISH) and indirect immunofluorescence (IF) staining can be used to detect the intracellular localization of endogenous mRNAs and proteins with fluorescently labeled oligonucleotide probes or antibodies, respectively. These techniques are not hampered by the difficulties

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encountered in the use of fluorescent proteins because they localize endogenous mRNAs and proteins, respectively. Chlorophyll autofluorescence can be eliminated when cells are chemically fixed and detergent-permeabilized. These steps required for FISH probes and antibodies to reach their intracellular targets. However, these techniques cannot reveal the dynamics of mRNA or protein localization in living cells and real-time, the major advantage of techniques involving GFP and other fluorescent protein tags.

Chlamydomonas has characteristic cellular anatomy and polarity (Fig. 1a) (9). The apical (anterior) pole is marked by the pair of flagella. The basal (posterior) cytosol is occupied mostly by a globular domain of the chloroplast from which a few finger-like lobes extend to the apical pole to cup a central nuclear-cytoplasmic region, containing the nucleus surrounded by cytoplasm, the endoplasmic reticulum and the Golgi apparatus (10–16). The chloroplast contains a prominent spherical body, the pyrenoid, which is located near the basal pole of every cell (Fig. 1a, f) (17). This highly stereotyped cellular anatomy facilitates the use of *Chlamydomonas* as a model organism for investigations of pattern formation at the cellular level (18).

This cellular anatomy also allows the researcher to identify the location(s) of a FISH or IF signal relative to prominent cytological landmarks (16, 18). The flagella, chloroplast, and pyrenoid are visible by light microscopy (Fig. 1b, c). The chloroplast also can be revealed by chlorophyll autofluorescence, when the researcher chooses not to eliminate it as described in Subheading 3.1, step 2 (19, 20). The nucleus (and chloroplast nucleoids) can be stained with DAPI (Fig. 2a, b), mitochondria with MitoTracker Green FM (Invitrogen, Carlsbad, CA, USA), and vacuoles with neutral red or MDY-64 (21).

Routine use of FISH and IF staining in *Chlamydomonas* promises to facilitate characterizations of the intracellular localization of proteins and mRNAs and bring unexpected discoveries. For example, the first FISH studies revealed surprisingly complex

Fig. 1. (continued) except for the cortical section in E (*lower row*). This cortical section was taken close to the cell perimeter and, therefore, it transects the lobe closest to the viewer. (**c**) The FISH signal of the nuclear–cytoplasmic *LhcII* mRNA, the IF signal of an r-protein of the cytoplasmic ribosome, and a DIC image of the same cell. (**d**) The *LhcII* mRNA FISH signal and the IF signal of the endoplasmic reticulum protein, protein disulfide isomerase (PDI). (**e**) The FISH signal of the chloroplast *psbA* mRNA and the IF signal of a thylakoid membrane protein, PsaA. *Arrows* indicate a gap in chloroplast lobe where it curves out of the optical section. Other cytosolic compartments extend into these gaps, as seen for PDI (and therefore endoplasmic reticulum) in *row d*. The cortical section shows that the chloroplast encloses the nuclear region as lobes rather than like the continuous rim of a cup. (**f**) The FISH of the chloroplast *rbcL* mRNA and the IF signal of Rubisco, a protein in the pyrenoid and chloroplast stroma. These results have been reported previously (16, 22, 23) (bar = 1 μm).

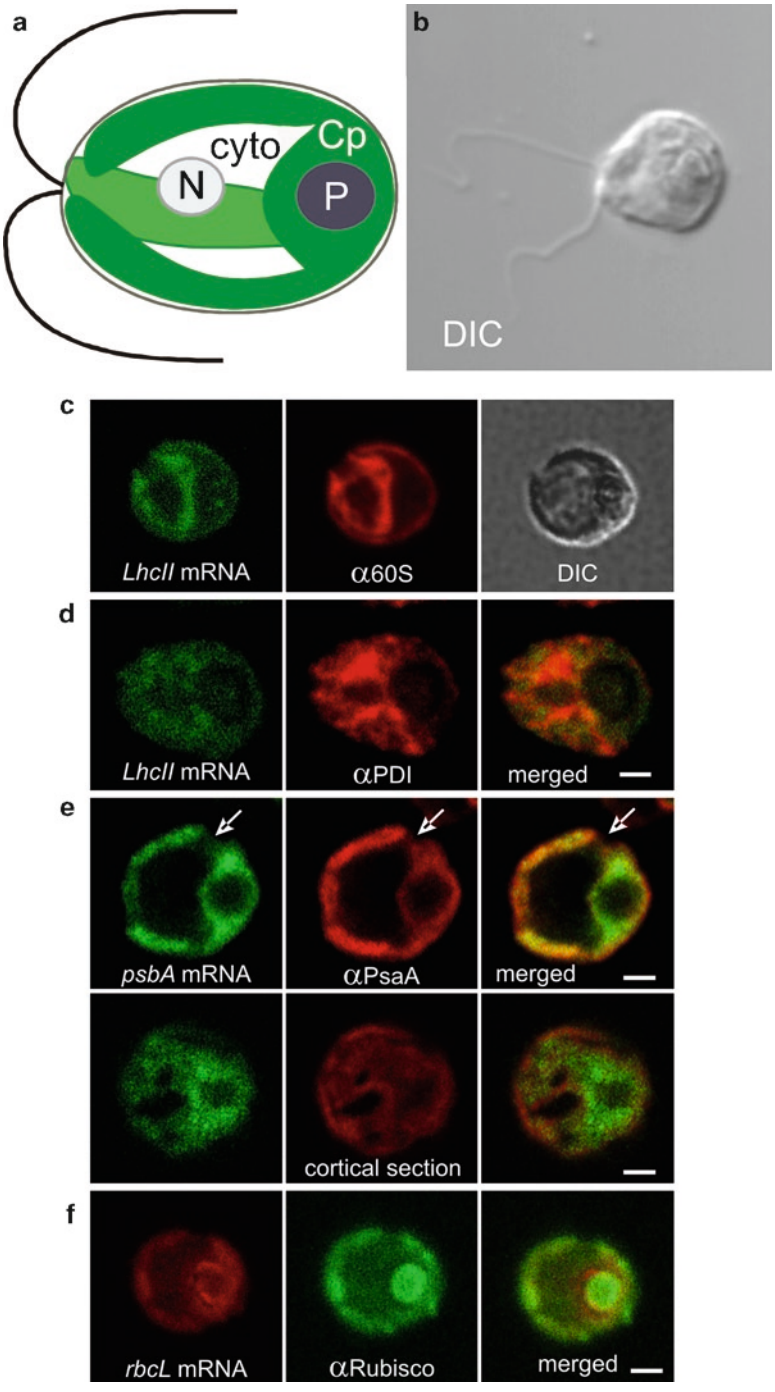

Fig. 1. *Chlamydomonas* cytology visualized by fluorescence microscopy. (**a**) An illustration of a cell oriented with its apical–basal (anterior–posterior) axis from left to right and showing the locations of the flagella, the nucleus (N), cytosol (cyto), the chloroplast (Cp), and the pyrenoid (P). (**b**) An image of a chemically fixed cell obtained by DIC microscopy. (**c**–**f**) Examples of FISH and IF signals (left-most and central columns, respectively) detected with a confocal laser-scanning microscope (Leica TCS SP2) in 0.2-mm longitudinal optical sections. Most optical sections were taken from the center of the cell,

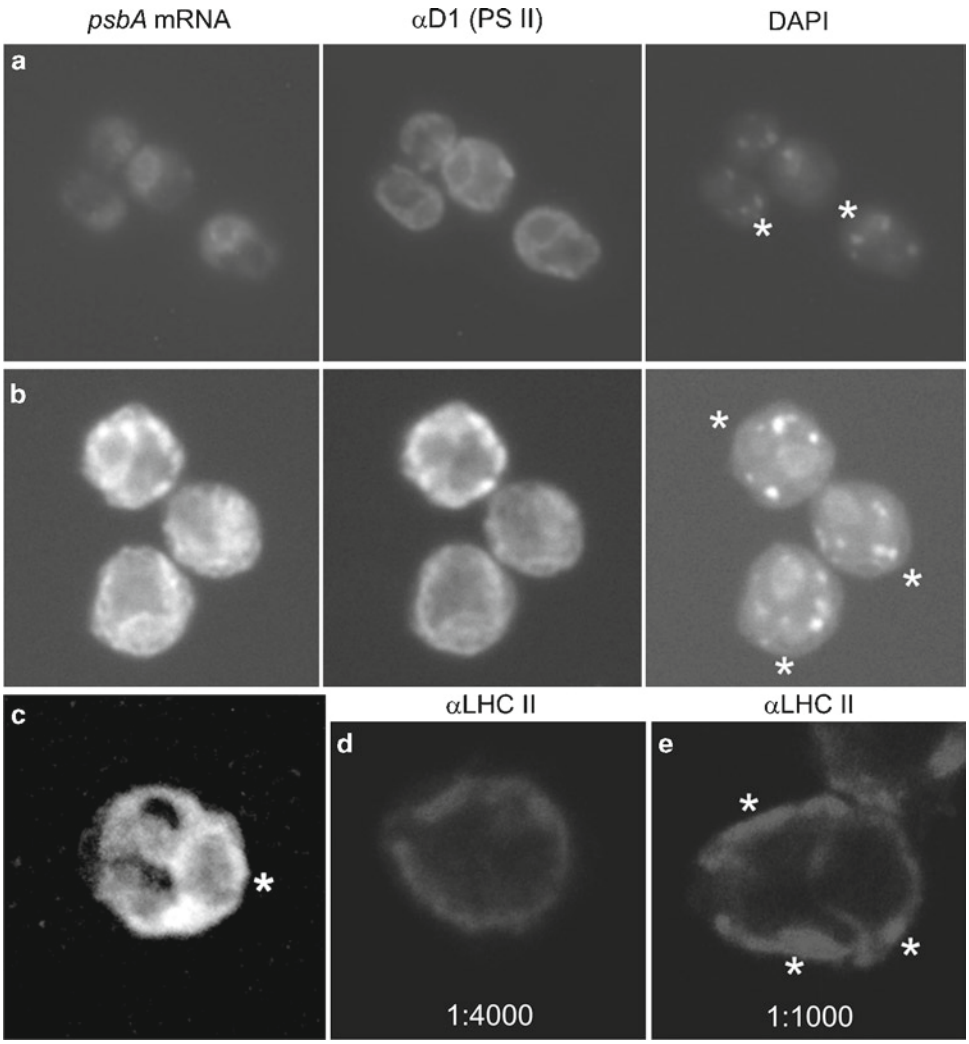

Fig. 2. Comparisons of images of the FISH and IF signals obtained from microscopes with different optic systems. (**a**–**c**) The FISH signal is from the *psbA* mRNA, the IF signal is from the photosystem II subunit D1, and DNA was stained with DAPI. (**a**) An axioplan fluorescence microscope (Zeiss). (**b**) Aristoplan microscope (Leitz) with Nomarski differential interference contrast (DIC) or epifluorescence optics. (**c**) A reconstructed cell image from a complete series of serial optical sections obtained with a confocal laser-scanning microscope (Leica TCS SP2). The basal (posterior) poles of most cells are marked with an *asterisks*. (**d**, **e**) The IF signal obtained from two different dilutions of antisera against the light harvesting complex II subunits (LHC). *Asterisks* in (**e**) indicate artifactual punctate IF signal seen when high antibody concentrations were used.

mRNA localization patterns for cellular pattern formation, protein targeting, and oxidative stress response (16, 18, 22, 23). Here we describe FISH and IF-staining protocols in detailed, yet streamlined fashion to facilitate their widespread use among *Chlamydomonas* researchers and cell biologists.

2. Materials

All chemicals and reagents should be analytical, bacteriological, or molecular biology grade.

2.1. Chlamydomonas Culture Media (24, 25)

1. Beijerinck salts (16 g NH_4Cl, 2 g $CaCl_2 \cdot 2H_2O$, and 4 g $MgSO_4 \cdot 7H_2O$ per liter, stored at 4°C).
2. Phosphate solution: 1.0 M KPO_4, pH 7 (250 mL of 1.0 M K_2HPO_4, approximately 170 mL of 1.0 M K_2HPO_4 to titrate to pH 7.0, stored at 4°C).
3. 40× Tris–Acetate Phosphate (TAP) medium stock; 96.8 g Tris-Base (Sigma), 40 mL 1 M of $KHPO_4$ to titrate to pH 7.0 with ca. 44 mL glacial acetic acid.
4. TAP medium: 25 mL of 40× TAP medium stock, 25 mL Beijerinck salts, and 1.0 mL Trace elements solution, per liter.
5. High-salt medium: 25 mL Beijerinck salts, 6.5 mL of 1 M $(K)PO_4$ pH 7.0, 1 mL of Trace elements solution.
6. Erlenmeyer flasks (50–200 mL).
7. Agar, bacteriological grade.
8. Ultra-pure water (marketed for high pressure liquid chromatography).

2.2. Trace Elements Solution

1. H_3BO_3.
2. $ZnSO_4 \cdot 7H_2O$.
3. $MnCl_2 \cdot 4H_2O$.
4. $FeSO_4 \cdot 7H_2O$.
5. $CoCl_2 \cdot 6H_2O$.
6. $CuSO_4 \cdot 5H_2O$.
7. $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.
8. Na_2EDTA.
9. 100 mL 20% KOH.

2.3. Cell Fixation and Permeabilization

1. Paraformaldehyde (toxic, purchased as a 20% (v/v) stock from Electron Microscopy Sciences, Hatfield, PA, USA; store at room temperature).
2. Methanol (Fisher), maintained at –20°C prior to use but can be stored at room temperature (toxic, inflammable).

2.4. FISH Probe Labeling

1. Synthetic oligonucleotides of 50 nt are designed and ordered, four for each target mRNA. Published protocols call for them to hybridize across exon junctions to promote specificity to the mature mRNA over unspliced precursors or genomic

DNA (16). We have used probes that hybridize within exons of a chloroplast mRNA that lack introns and these did not give a signal from chloroplast nucleoids, the structures that contain multiple copies of the chloroplast genome. The oligonucleotide sequences should be selected to have five T residues interspaced by 6–10 nt. At these positions, the oligonucleotide synthesis company is instructed to incorporate amine-modified C6-dT residues (26) (http://www.singerlab.org/protocols).

2. Amine-reactive fluorescent dyes as carboxylic acid, succinimidyl ester mixed isomers (Invitrogen). Fluorophores are selected based according to the compatibility of their excitation wavelengths with available microscope filters and lasers. We use Alexa Fluor 488 and Alexa Fluor 555 and Alexa Fluor 633 (see Notes 1 and 2). Shield from light whenever possible and store at –20°C.
3. 0.1 M Sodium bicarbonate at pH 9.0. Prepare 1 mL aliquots and store them at –20°C.
4. DMSO, store at room temperature.
5. 5.0 M NaCl, store at room temperature.

2.5. FISH Probe Purification by Denaturing Polyacrylamide Gel Electrophoresis

1. 0.1% Bromophenol blue in 50% formamide, prepared fresh.
2. 30% Acrylamide solution (19:1 acrylamide:bisacrylamide), store at 4°C.
3. 10% Ammonium persulfate, prepared fresh.
4. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), store at room temperature.
5. 5× TBE electrophoresis buffer (445 mM Tris Base, 445 mM Boric acid, 10 mM EDTA [pH 8.0]), store at room temperature.
6. Vertical electrophoresis apparatus, with glass plates of ca. 30 cm (width) × 40 cm (height), a comb and spacers (1 mm thickness).
7. High voltage power supply.
8. Hand-held UV light source.
9. Elution Buffer (0.1% SDS, 10 mM magnesium acetate, and 0.5 M ammonium acetate), phosphate buffer with salt (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4), PBS-Mg (PBS with 5 mM $MgCl_2$), prepared fresh.

2.6. Fluorescence In Situ Hybridization

1. Fingernail polish, store at room temperature.
2. 37°C Hybridization oven, such as a slide hybridization oven for microarrary hybridizations or a convection oven with precise temperature control.
3. 0.1% Poly-L-lysine (Sigma), store at room temperature.

4. 1 M HCl, store at room temperature.
5. 95% Ethanol, store at –20°C.
6. 70% Ethanol, store at room temperature.
7. Hemocytometer.
8. Coplin jars and lids.
9. ProLong Gold Anti-fade reagent (Molecular Probes).
10. 20× SSC (3.0 M NaCl, 300 mM sodium citrate, pH 7.0), store at room temperature.
11. 2.0 mg/mL Sheared salmon sperm DNA (Sigma). Store at –20°C.
12. 2.0 mg/mL *Escherichia coli* tRNA (Sigma). Store at –20°C.
13. Hybridization Buffer (4× SSC, 10 mM vanadyl ribonucleoside complex [VRC]).
14. 4.0 mg/mL bovine serum albumin (BSA), prepared fresh.
15. Prehybridization Buffer (2× SSC, 50% formamide), prepared fresh.
16. Posthybridization buffer (1× SSC, 50% formamide), prepared fresh.

2.7. Immunofluorescence Staining

1. IF staining requires the materials listed above, under Subheading 2.6, items 1–9.
2. Primary antibody. We have used rabbit polyclonal antibodies, as crude antisera and a mouse monoclonal antibody. Store in tightly sealed tubes at 4°C with 0.02% sodium azide (w/v) to prevent microbial contamination.
3. Secondary antibody is against the primary antibody and coupled to a fluorophore with an excitation wavelength that is compatible with filters or lasers on the available microscope(s). We use anti-rabbit or anti-mouse IgG–TRITC or IgG–FITC (Sigma).
4. PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4), store at room temperature. Blocking Solution: 0.1% BSA, and 2 mM VRC in 1× PBS, prepared fresh.
5. Primary and secondary antibody solutions (Blocking Solution with 10 mM VRC instead of 2 mM), prepared fresh.

3. Methods

The following control experiments should be considered. To determine the degree of signal specificity from a FISH probe set or antibody, cells deficient for the target mRNA or protein (e.g., due

to mutation, RNAi knock-down, or a known abiotic factor) can be analyzed in parallel to the experimental cells. If the preimmune serum is available, it can be tested as a negative control. None of several different commercial secondary antibodies that we have used generated an above-background fluorescence signal in the absence of primary antibody. Nevertheless, the secondary antibody alone could be excluded as a negative control. To reveal any background autofluorescence, the FISH probe or antibodies can be excluded from a sample. It should be noted that excitation at 633 nm generates a punctuate autofluorescence within the pyrenoid. Most excitation wavelengths generate chlorophyll autofluorescence if the steps in Subheading 3.3, step 5 are not properly carried out.

Fluorescence microscopy is beyond the scope of this article (27). However, we wish to note that optical sectioning with a confocal microscope allows the researcher to normalize signal intensity across compartments with different volumes. For example, in whole cell images obtained with an epifluorescence microscopy, the greater depth of the field can make FISH and IF signals falsely appear highly concentrated in the chloroplast basal region due to its greater volume, relative to the chloroplast lobes (data not shown). Figure 2a–c show examples of images obtained by epifluorescence, Nomarski differential interference contrast (DIC), and confocal laser scanning microscopes.

3.1. Chlamydomonas Cell Culture (9, 24, 25)

The use of a cell wall (CW) mutant strain is advantageous because cells are generally larger and rounder than wild type and some pharmacological agents are ineffective in wild-type cells. We use CC-503 which carries *CW94*. However, it is advisable to also use wild type to control for potentially aberrant localization patterns in CW mutants. Cells can be cultured under photoautotrophic conditions on HSM medium in the light, mixotrophic conditions on acetate-containing TAP medium in the light. These conditions generate very different cell types (28). For analyses of localization patterns of mRNAs or proteins related to photosynthesis, we advise the use of cells cultured under photoautotrophic conditions whenever possible.

When water quality is of doubt or the cells seem to be in suboptimal condition, it may be helpful to use ultra-pure water (for high pressure liquid chromatography) for liquid media and for solutions to which cells are exposed:

1. Media are prepared with ingredients listed in Subheading 2.1 and sterilized by autoclaving.
2. Growth conditions are at 24°C with illumination by one or more 20 W fluorescent bulbs marketed for plants at a distance of 10–20 cm.
3. Strains are maintained on medium solidified with 1.5% bacteriological grade agar (wt/vol) in petri plates and transferred

to fresh plates every 3 days prior to inoculation of the liquid culture(s).

4. A liquid culture of 25–50 mL in 100 mL Erlenmeyer flask is grown over 1–2 days from an initial density of ca. 10^4 cells/mL to a final density of $2–5 \times 10^6$ cells/mL, as determined with a hemocytometer.

3.2. Trace Elements Solution (25)

1. In 550 mL H_2O, dissolve in order; 11.4 g H_3BO_3, 22 g $ZnSO_4 \cdot 7H_2O$, 5.06 g $MnCl_2 \cdot 4H_2O$, 4.99 g $FeSO_4 \cdot 7H_2O$, 1.61 g $CoCl_2 \cdot 6H_2O$, 1.57 g $CuSO_4 \cdot 5H_2O$, and 1.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.
2. Dissolve 50 g Na_2EDTA in 250 mL H_2O by heating to near 100°C.
3. Mix the two solutions and bring the resulting solution to boiling.
4. Cool to 80–90°C and adjust to pH 6.5–6.8 with ca. 100 mL 20% KOH using a pH meter calibrated at 75°C.
5. Adjust volume to 1 L with H_2O and incubate at room temperature for approximately 2 weeks in a 2 L-Erlenmeyer flask loosely plugged with cotton. A precipitate forms.
6. Filter through filter paper (Whatman).
7. Make aliquots of 50–200 mL. The working aliquot is stored at 4°C. Reserves are stored at −20°C.

3.3. Cell Fixation and Permeabilization

1. To reduce autofluorescence of the microscope slides, they are boiled in 1 M HCl for 15 min, air-dried, covered with aluminum foil, and incubated at least overnight at room temperature. Take appropriate safety precautions for manipulation of acid.
2. To enhance adherence of the cells to slides in the next step, 10 µL of 0.1% Poly-L-lysine is dispensed near one end of each slide and then smeared across its length using the edge of another slide. Treated slides are stored at room temperature in a slide rack covered with aluminum foil for 3–7 days.
3. An aliquot of ca. 500 µL containing ca. 10^6 cells is dispensed to the center of a poly-L-lysine-coated microscope slide. Cells are allowed to adhere to the slide for 45 s. One must keep track of the side of the slide with the cells prior to mounting in step 3 (Subheading 3.1) because ink labels are not resistant to the intervening steps.
4. Cells are fixed by incubating the slide for 10 min at room temperature in a Coplin jar containing 4% paraformaldehyde (v/v) freshly diluted in PBS (see Note 2).
5. Slides are incubated twice in methanol for 10 min at −20°C, again in a Coplin jar (see Note 3).

6. Slides are given two 10 min washes in PBS-Mg at room temperature.
7. Cell permeabilization involves incubating the slide in freshly prepared 2% (v/v) Triton X-100 in PBS for 10 min at room temperature (see Note 4).
8. Slides are given two additional 10 min washes in PBS-Mg at room temperature.
9. Slides are ready for FISH (Subheading 3.6) or IF staining (Subheading 3.7).

3.4. Labeling of FISH Probes

These procedures for labeling and purification of oligonucleotide FISH probes have been reported previously (26) (http://www.singerlab.org/protocols, http://probes.invitrogen.com/media/pis/mp00143.pdf) (see Note 5):

1. Labeling reactions contain 4 µL of 25 µg/µL oligonucleotide (with the five amine-modified C6-dT residues), 14 µL with 250 µg of amine-reactive fluorophore (resuspended in DMSO), 75 µL 0.1 M sodium bicarbonate buffer (pH 9.0), and 7 µL deionized water (see Note 6).
2. This labeling reaction is incubated overnight, in the dark and at room temperature.
3. The oligonucleotide is precipitated by the addition of 10 µL 5 M NaCl solution and 250 µL cold 95% ethanol, mixed, incubated at −20°C for 30 min, and finally centrifuged at maximum speed, e.g., 14,000 × *g* for 30 min at 4°C.
4. The pellet is washed with 75% ethanol and centrifuged at maximum speed, e.g., 14,000 × *g* for 2 min.
5. The pellet is dried by leaving the tube open for ca. 10 min.

3.5. FISH Probe Purification by Denaturing Polyacrylamide Gel Electrophoresis

The labeled oligonucleotide is purified from unlabeled oligonucleotide and free dye by denaturing polyacrylamide gel electrophoresis:

1. Mix 13 mL of a 30% acrylamide/bisacrylamide solution, 6 mL of 5× TBE buffer, 10.68 mL of water, 100 µL ammonium persulfate, and 24 µL TEMED.
2. Immediately dispense this solution between the glass plates with a 25 mL pipet.
3. Insert comb and allow the gel to polymerize for at least 30 min.
4. Prepare TBE electrophoresis buffer by diluting 200 mL of 5× TBE buffer in 800 mL water.
5. After the gel has polymerized, carefully remove the comb and use a 3-mL syringe fitted with a 22-gauge needle to rinse the wells with TBE electrophoresis buffer.

6. Attach the gel to the electrophoresis apparatus.
7. Add the TBE electrophoresis buffer to the upper and lower reservoirs.
8. The gel is exposed for 30 min to a power of 30–50 W and adjusted to maintain the glass plates at approximately 55°C. Exercise caution appropriate for high voltages.
9. Labeled oligonucleotide probes, from Subheading 3.4, step, are resuspended in 0.1% bromophenol blue in 50% formamide to the volume of a well of the gel (e.g., 50 μL).
10. Samples are incubated at 55°C for 5 min and loaded into lanes of the gel.
11. Electrophoresis is carried out at the wattage determined in Subheading 3.5, step 8 and until the bromophenol blue has migrated 60–70% of the gel. The labeled oligonucleotide should be faintly visible as a colored band during electrophoresis.
12. The power supply is turned off.
13. The gel is removed from the apparatus.
14. The glass plates are separated to expose the gel.
15. The band with the labeled oligo is visualized in a dark room with a hand-held UV source and excised from the gel with a razor blade. Labeled oligonucleotide will have migrated approximately to the middle of the gel while the free dye will have migrated with the bromophenol blue.
16. The excised gel fragment is transferred to a 1.5 mL microfuge tube, manually crushed (e.g., with a micropipette tip), and incubated overnight in elution buffer at 37°C with agitation. The probe is shielded from light whenever possible to prevent photobleaching of the fluorophore. Our probe solutions have 50–100 ng oligonucleotide/mL and a frequency of incorporation of 50–70 flour molecules per 1,000 bases, 3.1 flour molecules/probe (see Note 7).

3.6. Fluorescence In Situ Hybridization (see Notes 8 and 9)

1. The amount of FISH probe used may have to be determined empirically for each target mRNA. For each hybridization reaction, we prepare a solution containing 30–50 ng of probe and 2 μg each of sheared salmon sperm DNA and *E. coli* tRNA (from stock solutions of 2 μg/μL, stored at −20°C).
2. This solution is lyophilized at 43°C to dryness, resuspended in 10 μL of 100% ultrapure formamide, and then incubated at 85°C for 5 min.
3. A 10 μL aliquot of this hybridization solution is dispensed to the center of a cover slip situated on a piece of parafilm long enough to hold all the microscope slides. The probe mixture is then dispensed into this droplet of hybridization buffer.

4. Each slide is gently blotted with KimWipes and placed with the cell-side down onto a cover slip. Ideally, hybridizations are carried out in a slide hybridization oven with a piece of moist paper towel to maintain humidity. If a convection oven is used, slides are placed on a glass plate, which is then sealed with parafilm to prevent the hybridization solutions from drying. If the shelf of the convection oven has holes, something is placed on them to prevent air circulation in the immediate vicinity of the slides. We generally carry out hybridizations at 37°C when probing for chloroplast mRNAs and at 42°C for nuclear–cytosolic mRNAs. It may be necessary to test a range of hybridization temperatures for each target mRNA to optimize the signal to background ratio. Hybridizations are carried out overnight.
5. For each five-slide Coplin jar, 100 mL of posthybridization buffer (1× SSC, 50% formamide) is freshly prepared and incubated for approximately 30 min at 37°C.
6. Slides are washed twice for 20 min in this buffer at 37°C.
7. Slides are washed for 10 min at room temperature in 0.5× SSC.
8. Slides are washed for 10 min at room temperature in 0.25× SSC.
9. Slides are washed for 10 min at room temperature in 1× PBS, 5 mM $MgCl_2$.
10. Slides are gently blotted dry with KimWipes and 20 µL of ProLong Gold Anti-fade reagent is dispensed onto the cells.
11. A cover slip is placed on the slide and the edges are sealed with fingernail polish. The cells can viewed immediately, however, an overnight incubation at room temperature in the dark seems to improve fluorescence signal strength. Slides can be stored for at least 1 year at –20°C without noticeable loss of signal.

3.7. IF Staining

IF staining can be initiated after Subheading 3.3, step 9 or when used in combination with FISH, immediately after Subheading 3.6, step 9:

1. A 25 µL aliquot of blocking solution (PBS, 0.1% BSA, and 2 mM VRC) is dispensed onto a cover slip.
2. The slide with the fixed and permeabilized cells is dried by gentle blotting with KimWipes and then placed cell-side down onto the cover slip with the blocking solution.
3. The slide is incubated for 30 min at room temperature.
4. The cover slip is removed and cells are then incubated under a new cover slip in primary antiserum diluted with blocking buffer (containing 10 mM VRC) at 37°C for 75 min. The optimal dilution factor must be determined empirically for each primary antibody. We dilute most primary antisera at

1:1,000 although some must be diluted at 1:4,000 (see Note 10, Fig. 2d, e).

5. The cover slip is removed and slide is then washed twice in PBS for 10 min at room temperature.
6. Cells are incubated under a new cover slip in a 1:200 dilution of secondary antibody in blocking buffer (with 10 mM VRC) at room temperature for 45 min.
7. The cover slip is removed and the slide is washed in PBS for 10 min at room temperature.
8. Slides are blotted dry with KimWipes and a 25 μL drop of ProLong Gold Anti-fade is dispensed at the center of the slide and a new coverslip is placed over the drop.

4. Notes

1. Samples can be FISH-probed concurrently for multiple mRNAs using fluorescent dyes with as widely separated emission/excitation spectra as possible. The number of dyes that can be used is limited by the available laser lines. We have simultaneously probed for three mRNAs using Alexa Fluor 488, 555, and 633 dyes.
2. Fixation with glutaraldehyde results in high background autofluorescence.
3. The methanol treatments must be carried out entirely at −20°C to eliminate chlorophyll autofluorescence. These treatments can be omitted to retain chlorophyll autofluorescence in order to "stain" the chloroplast.
4. Poor permeabilization by Triton X-100 can prevent IF staining (Fig. 2b). We found that a Triton X-100 concentration of 2% (v/v) is required.
5. Other FISH probe systems exist, which we have not used. One is reported to allow quantitative expression analysis, single-copy mRNA detection, and does not require the laborious steps in Subheading 3.1, step 1 (QuantiGene ViewRNA, Affymetrix, Fremont, CA) (29). Another system has been used in *Chlamydomonas*. Oligonucleotide probes are labeled with digoxigenin or biotin, which are detected by immunofluorescence (30).
6. The labeling reaction must not contain Tris because it reacts with amine reactive dyes. Invitrogen's labeling protocol calls for 0.1 M sodium tetraborate (pH 8.5) instead of 0.1 M sodium bicarbonate (pH 9.0) (Invitrogen Manual "Amine Reactive Probes" at http://probes.invitrogen.com/media/pis/mp00143.pdf).

7. The NanoPhotometer (Implen) determines the oligonucleotide concentration and FOI of a probe using an aliquot of only 1 µL. See also Invitrogen's Manual "Amine Reactive Probes" (http://probes.invitrogen.com/media/pis/mp00143.pdf).
8. FISH signals of chloroplast mRNAs are stronger than those of even very abundant nuclear–cytosolic mRNAs.
9. A minority of experimental trials using FISH give only a weak signal that is dispersed throughout the cell. This problem is more commonly encountered when probing for nuclear–cytosolic mRNAs than for chloroplast mRNAs.
10. Excess antibody, particularly those against abundant proteins, can block central IF staining and generate artifactual punctate staining patterns (Fig. 2, compare d and e). Therefore, the primary antibody is titred by analyzing the signal strength and in situ localization patterns obtained with a range of primary antibody dilutions (e.g., from 10^{-2} to 10^{-4}). Subsequent experiments use the lowest concentration that gives an adequate quantifiable signal.

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