Conjugation of Fluorescent Probes to Proteins and Nucleic Acids

Marty R. Jacobson, Thoru Pederson, and Yu-Li Wang

I. INTRODUCTION

Microinjection of fluorescent conjugates of proteins and nucleic acids has been used as a powerful approach to study molecular dynamics in living cells (see also other articles in Volume 4 for additional information). Imaging of injected probes allows the direct observation of structural assembly and reorganization. In addition, analysis with resonance energy transfer (Adams *et al.*, 1991), photobleaching (Wang, 1985), and photoactivation (Theriot and Mitchison, 1991) techniques can provide insights into molecular conformation and interactions in living cells.

The preparation of fluorescent probes represents a critical first step in this approach. It typically involves the covalent reaction of purified target molecules with a fluorescent reagent, which consists of a fluorophore with an associated reactive group. This article first describes a general method for the preparation of fluorescent RNA (and DNA). Because the procedure of protein conjugation can vary dramatically with reagents and proteins, we use the preparation of tetramethylrhodamine α-actinin as an example, with additional notes given at each step to cover some general principles and considerations. Readers are referred to Wang (1989, 1992) for additional discussions on the preparation of protein conjugates.

II. MATERIALS AND INSTRUMENTATION

A. For Fluorescent Conjugation of RNA

Major suppliers of bacteriophage RNA polymerases (T7, T3, and SP6) used in *in vitro* transcription reactions include GIBCO-BRL, New England Biolabs, Promega, and Stratagene. Dithiothreitol (DTT; Cat. No. 100-032) is from Boehringer Mannheim. ATP, CTP, GTP, and UTP (Cat. Nos. 27-2056-01, 27-2066-01, 27-2076-01, and 27-2086-01, respectively) and Sephadex G50 (Cat. No. G-50.80) are from Pharmacia. ^{7me}GpppG (cap analog; Cat. No. 1404) is from New England Biolabs. 5-(3-Aminoallyl)UTP (Cat. No. A-5660), *N,N*-dimethylformamide (DMF; Cat. No. 27054-7), dimethyl sulfoxide (DMSO; Cat. No. D-2650), Trizma acetate (Cat. No. T-1278), and Trizma HCl (Cat. No. T-3253) are from Sigma. *N*-(6-Aminohexyl)ATP (Cat. No. 19516-012) is from GIBCO-BRL. RNasin (20–40 units/μl; Cat. No. N2511) and RQ1 DNase (RNase free; 1 unit/μl) are manufactured by Promega (Cat. No. M6101). Tetramethylrhodamine-5-isothiocyanate (TRITC; Cat. No. T-1481) and fluorescein-5-isothiocyanate (FITC; Cat. No. F-143) are from Molecular Probes. BioGel P60 (Cat. No. 150-4160) is from Bio-Rad. All chemicals are at least reagent grade. The protocol

also requires a table-top microfuge, a heating block, a rocking platform, a UV spectrophotometer, and a vertical gel apparatus for RNA.

B. For Fluorescent Conjugation of α-Actinin

PIPES (Cat. No. 736-953) and DTT (Cat. No. 100-032) are from Boehringer Mannheim. Tris acetate (Cat. No. T-1258) is from Sigma. Tetramethylrhodamine-5-iodoacetamide (TRIA; Cat. No. T-6006) is from Molecular Probes. Bio-Beads SM2 (Cat. No. 152-3920) is from Bio-Rad and is packed into a 0.7 × 15-cm column. Centricon-30 concentrators (Cat. No. 4208) are from Amicon. Also required are an ultracentrifuge and rotors/adapters for small volumes, e.g., Beckman type 42.2 Ti rotor, a spectrophotometer, reagents for the Lowry protein assay, and an apparatus for protein PAGE.

III. PROCEDURES

A. Preparation of Fluorescently Conjugated RNA

Solutions

Make sure that all materials used for RNA synthesis and purification are sterile and RNase free.

1. *Buffer A:* 10 m*M* Tris–HCl, pH 7.0. Prepare 1.0 *M* stock (stir 12.1 g Trizma Base in ~80 ml of distilled H_2O and adjust pH with concentrated HCl, then bring up the volume to 100 ml), dilute 1:100.

2. *Buffer B (5× transcription buffer):* 200 m*M* Tris–HCl (pH 7.5), 30 m*M* $MgCl_2$, 10 m*M* spermidine, and 50 m*M* NaCl. Prepare a 1.0 *M* Tris–HCl, pH 7.5, stock (stir 12.1 g Trizma Base in ~80 ml of distilled H_2O and adjust to pH 7.5 with concentrated HCl, then bring up the volume to 100 ml). To ~70 ml of distilled H_2O, add 20 ml of 1 *M* Tris–HCl (pH 7.5), 0.286 g $MgCl_2$, 0.145 g spermidine, and 0.292 g NaCl, stir, and bring the volume to 100 ml.

3. *10× buffer C:* 1.0 *M* sodium carbonate–bicarbonate (pH 9.0). Prepare 1.0 *M* sodium carbonate (8.4 g dissolved in 100 ml of distilled H_2O, final volume) and 1.0 *M* sodium bicarbonate (10.6 g dissolved in 100 ml of distilled H_2O, final volume). Add the 1.0 *M* sodium carbonate to the 1.0 *M* sodium bicarbonate to pH 9.0 (~1:10 ratio).

4. *Buffer D:* 5 m*M* Tris–acetate (pH 7.0). Prepare a 1.0 *M* stock (stir 18.1 g Trizma Acetate in ~80 ml distilled H_2O, adjust pH to 7.0, then bring the volume to 100 ml), dilute 1:200.

5. *10× rNTP mix:* 10 m*M* ATP, 10 m*M* CTP, 10 m*M* GTP, and 10 m*M* UTP. Add 100 μl each of 100 m*M* ATP, 100 m*M* CTP, 100 m*M* GTP, and 100 m*M* UTP stock solutions (supplied by the manufacturer) to 600-μl sterile distilled H_2O. Store frozen.

6. *10 mM ^{7me}GpppG:* Add 137 μl sterile, RNase-free H_2O to 25 OD units (21.5 OD/mg obtained from the supplier).

7. *1.0 mM 5-(3-aminoallyl)UTP:* Dissolve 5.39 g in 1.0 ml of distilled H_2O. Note that this can be substituted with 1.0 m*M* *N*-(6-aminohexyl)ATP, which is prepared by diluting the 10 m*M* stock solution (supplied by the manufacturer) 1:10 in sterile distilled H_2O. Store frozen.

8. *RNasin (20–40 unit/μl):* As supplied by the manufacturer.

9. *RQ1 DNase (1 unit/μl):* As supplied by the manufacturer.

10. *0.1 M DTT:* Dissolve 1.54 g in 100 ml distilled water at 4°C. Store aliquots frozen.

11. *3 M sodium acetate (pH 5.2):* Dissolve 40.8 g of soidium acetate in ~80 ml distilled H_2O, titrate with concentrated acetic acid to pH 5.2, and adjust the final volume to 100 ml.

12. *Ethanol (absolute).*

13. *Phenol/chloroform/isoamyl alcohol (PCI; 25/25/1):* Mix 25 ml phenol, 25 ml chloroform, and 1 ml of isoamyl alcohol; store refrigerated.

14. *Water (sterile, RNase-free).*

Steps

1. *Preparation of the DNA template:* Linearize the plasmid DNA to be transcribed using the appropriate restriction enzyme. If the restriction enzyme used leaves a 3′ overhang, it should be removed using the 3′→5′ exonuclease activity of Klenow DNA polymerase (Sambrook *et al.*, 1989). Remove residual nucleases by extracting with an equal volume of PCI. After PCI extraction, transfer the aqueous phase to a sterile, RNase-free microcentrifuge tube, add 0.1 vol of 3 *M* sodium acetate (pH 5.2) plus 2–3 vol of ethanol, and incubate at −20°C for 1 hr. Centrifuge at top speed for 10 min in a table-top microfuge to pellet the DNA. Remove the ethanol and dry the DNA pellet. Resuspend the pellet in buffer A to a concentration of 1 mg/ml and store at −20°C.

2. In vitro *transcription of 5-(3-aminoallyl)uridine substituted RNA (AA-RNA):* Bring all reagents, buffers, etc. (except enzymes) to room temperature. In a microcentrifuge tube, add the following to total 500 μl: 185 μl H_2O, 100 μl buffer B, 20 μl 0.5 *M* DTT, 20 μl RNasin, 50 μl 10× rNTP mix, 50 μl 10 m*M* ^{7me}GpppG, 50 μl 1 m*M* 5-(3-aminoallyl)UTP, 20 μl linearized plasmid DNA (1 mg/ml), and 5 μl RNA polymerase (T7, T3, or SP6; 15–20 units/μl). Gently mix the contents in the tube and incubate the reaction mix at 37°C for 1–2 hr. Add 5 μl of RQ1 DNase and incubate for an additional 10 min to remove plasmid DNA. Heat inactivate the reaction at 65°C for 10 min and then cool on ice for 10 min. To precipitate the *in vitro*-transcribed RNA, add 0.1 vol (50 μl) of 3 *M* sodium acetate (pH 5.2) plus 2 vol (1000 μl) ethanol, mix, incubate at −20°C for 1 hr (or on dry ice ~10 min), and centrifuge for 10 min at top speed in a table-top centrifuge. Remove the ethanol and dry the pellet. Resuspend the AA-RNA in 150 μl H_2O. The resulting concentration should be approximately 1.0 μg/μl.

In vitro transcription kits are available from suppliers such as Ambion and are optimized for the yield of RNA. They can be used for the synthesis of aminoallyl-uridine-substituted RNAs.

In addition to ^{7me}GpppG, other RNA cap analogs (e.g., GpppG, GpppA, ^{7me}GpppA) are available from New England Biolabs if needed. Because many cellular RNAs (RNA polymerase I and most RNA polymerase III transcripts) are transcribed without a cap, the type of RNA to be synthesized should be considered prior to including cap analogs in the *in vitro* transcription reactions.

3. *Fluorescent labeling of* in vitro*-transcribed AA-RNA:* Resuspend the fluorescent dye of choice (FITC or TRITC) in *N,N*-DMF to a final concentration of 10 mg/ml. Dimethyl sulfoxide can be substituted for *N,N,*-DMF. In a microcentrifuge tube, add the following for a total of 300 μl: 150 μl AA-RNA (from the transcription above), 30 μl 10× buffer C, 50 μl fluorescent dye (10 mg/ml in *N,N*-DMF), and 70 μl H_2O (sterile, RNase-free). Incubate for 4–16 hr in the dark at ~20°C in a tube mounted on a rocking platform. If precipitation occurs during the coupling, the final concentration of DMF or DMSO can be increased up to 40% of the volume of reaction mixture. After the coupling reaction is completed, purify the fluorescently labeled RNA from free dye by gel filtration through either a 10-ml Bio-Gel P60 column or a Sephadex G50 column, eluting with sterile H_2O. Pool the peak RNA fractions from the column and concentrate by adding 0.1 vol of 3 *M* sodium acetate (pH 5.2) plus 2 vol of ethanol, incubating at −20°C for 1 hr, and centrifuging at top speed in a table-top microfuge for 10 min. Remove the ethanol from the pellet and resuspend in 400 μl of buffer D. Repeat ethanol precipitation and resuspend the fluorescently labeled RNA in buffer D to a final concentration of approximately 50 μg/ml. If necessary, RNAs can be further purified by denaturing gel electrophoresis (Sambrook *et al.*, 1989).

4. Check an aliquot of the RNA by denaturing gel electrorphoresis in an 8% polyacrylamide gel containing 8.3 *M* urea (Sambrook *et al.*, 1989). When the gel is illuminated on a short-wave ultraviolet (254 nm) light box there should be a single fluorescent band at a position corresponding to the RNA. Determine the RNA concentration by A_{260} (OD 1.0 corresponds to 33 μg/ml of single-stranded RNA).

5. Determine the amount of coupled dye by measuring absorbance at its peak excitation wavelength. FITC is measured at 495 nm and has a molar extinction coefficient of 76,000. TRITC is measured at 560 nm and has a molar extinction coefficient of 94,000. The number of fluorophores per RNA molecule is then calculated from their estimated molar concentrations.

B. Conjugation of α-Actinin with Tetramethylrhodamine

Solutions

1. *2 mM Pipes (pH 7.0):* Prepare 100 m*M* stock (stir 3.0 g in ~80 ml distilled water and adjust pH to 7.0 with concentrated KOH; bring up the volume to 100 ml), dilute 1:50.

2. *2 mM Tris–HCl (pH 8.5):* Prepare 500 m*M* stock of Tris–HCl (dissolve 7.9 g Trizma HCl in ~90 ml 4°C distilled water, adjust pH to 8.5, then bring up the volume to 100 ml), dilute 1:250.

3. *200 mM K-borate (pH 9.0):* Dissolve 6.2 g boric acid in ~480 ml distilled water, titrate with KOH to pH 9.0, and adjust the volume to 500 ml.

4. *100 mM DTT:* Dissolve 1.54 g in 100 ml distilled water at 4°C. Store aliquots frozen.

Steps

1. Dialyze 2 mg gizzard α-actinin at 5 mg/ml against 2 m*M* PIPES, pH 7.0, at 4°C. In general, proteins are dialyzed overnight into a solution that maintains their activity without interfering with the reaction. Tris and DTT should be avoided in the dialysis buffer for most conjugation reactions. A low concentration of buffer should be used if the conjugation is to take place at a different pH. If the sulfhydryl group is the target, the protein should be treated with 1–5 m*M* DTT for 30 min on ice, followed by dialysis against a DTT-free buffer. Thereafter the solution should be handled very gently to avoid oxidation.

2. Resuspend 1 mg TRIA in 150 μl acetone in a small glass vial. Grind any large particles against the side of the vessel with the tip of a Pasteur pipette until the dye forms a fine slurry. In general it is much easier to dissolve the reagent by first dissolving/suspending it in an organic solution before bringing it to the aqueous environment. Different solvents may be tried for this purpose, e.g., dimethylformamide, acetone, and methanol.

3. Add 1 ml of the borate buffer very slowly to the slurry of dye, with constant stirring. Borate buffer is ideal for reactions at pH >8.0. A relatively strong buffer should be used since many reactions affect the proton concentration.

4. Clarify the TRIA solution at 100,000 *g*, 4°C, for 10 min.

Steps 2–4 yield a solution of the reagent in a buffer appropriate for reaction, containing a limited amount of organic solvent. This procedure works for reagents with a reasonable solubility. If no visible pellet is found after centrifugation, step 4 can be deleted. On the other hand, if most of the reagent appears to be removed by centrifugation, it would be better to skip step 4 and carry out the reaction as a turbid mixture. However, in this case the yield and/or the extent of the reaction may be reduced. An alternative method is to dissolve the reagent in an organic solvent and mix with diatomaceous earth (Sigma Cat. No. D-5384). The solvent is then removed by evaporation, and the coated particles are mixed with the protein. Following the reaction the particles are removed by centrifugation.

5. Remove the top part of the dye solution from the centrifuge tube. Mix equal volumes of the reagent and α-actinin in a test tube by pipetting. Cover the vessel with Parafilm and foil.

6. Incubate on ice for 2 hr in the dark. The optimal temperature and time of reaction should be determined for each protein and reagent by trial and error. However, to adjust the extent of conjugation, it is usually more effective to adjust the concentration of the reagent or the pH of the buffer.

7. Equilibrate SM2 column with 2 m*M* Tris–HCl, pH. 8.5, at 4°C. The SM2 column works very well for removing unreacted tetramethylrhodamine reagents, but not for other fluorophores that we have tested. Although a G25 column can be used for most reagents,

some of them form microscopic aggregates that move in the excluded volume of the G25 beads. In addition, some fluorophores adsorb to the protein and cannot be separated with a G25 column. Thus the effectiveness of the column should always be checked with PAGE (described later). Generally it is difficult to thoroughly remove unreacted dye by dialysis.

8. Bring the concentration of DTT in the reaction solution to 10 m*M* by adding 1/9 volume of the 100 m*M* stock. Clarify the solution at 100,000 *g*, 4°C, for 20 min. Although it is not always essential to quench the reaction (by DTT for sulfhydryl-directing reagents, or glycine for amine-reacting reagents), this appears to facilitate the removal of unreacted reagents.

9. Load the supernatant to the SM2 column. Unreacted tetramethylrhodamine binds to the SM2 beads whereas protein conjugates flow through rapidly. Pool fractions that appear visibly pink.

10. Concentrate the protein with a Centricon-30 to 5–10 mg/ml.

11. Check the conjugate by standard SDS–PAGE. Stop the electrophoresis before the tracking dye runs off the gel. Examine the gel with a hand-held UV lamp or an UV transilluminator. There should be no visible fluorescence near the front marked by the tracking dye. All of the fluorescence should colocalize with the protein target.

12. Determine the concentration of the protein with the Lowry assay. Other assays may be used as long as the measuring wavelength does not overlap with the absorption wavelength of the fluorophore. Measurements based on absorbance at 280 nm is less reliable due the absorption of UV by most fluorescent dyes.

13. Determine the concentration of conjugated fluorophore by measuring the absorbance at its peak absorption wavelength. It may be necessary to dilute an aliquot of the concentrated protein solution to obtain a reliable reading. The molar extinction coefficient can be obtained from the literature, the manufacturer, or from actual measurements. For tetramethylrhodamine, we use a molar extinction coefficient of 60,000 at 555 nm. The number of fluorophores per protein molecule can then be calculated based on the molar concentrations of protein and the fluorophore.

C. Preparation for Microinjection

(See other articles in Volume 4 for additional information).

Solution

1. *5 mM Tris–acetate, 0.1 mM DTT (optional) (pH 7.0):* Prepare 500 m*M* stock of Tris–acetate (dissolve 9.05 g in ~90 ml distilled water at room temperature, adjust pH to 7.0, then bring up the volume to 100 ml), dilute 1:100.

Steps

1. Proteins should be (micro)dialyzed overnight against Tris–acetate, which is found to cause minimal cell damage during needle microinjection.

2. Before needle microinjection, it is important to remove all the aggregates from the solution. This is typically achieved by centrifugation at 100,000 *g*, 4°C, for 20 min. Remove the top portion of the supernatant carefully for microinjection. Immediately before microinjection, fluorescently labeled RNA should be heated for ~5 min at 75°C to denature the RNA molecules.

IV. COMMENTS

In addition to the protocol described in Procedure A, fluorescently coupled rNTP, available from Boehringer Mannheim and DuPont/NEN, can be used in *in vitro* transcription reactions, thereby avoiding the coupling step. However, these fluorescently labeled rNTPs are incor-

porated by RNA polymerases significantly less efficiently than the corresponding unlabeled rNTPs.

Fluorescently labeled DNA can also be synthesized by the incorporation of either amino-containing dNTPs or commercially available fluorescent-dNTPs. In contrast to RNA polymerases, DNA polymerases efficiently incorporate substituted dNTPs in standard nick translation or end-labeling reactions (Langer *et al.*, 1981; Sambrook *et al.*, 1989). In addition, amino-containing oligonucleotides can be synthesized and coupled with fluorophores using the coupling methods described earlier (Taneja *et al.*, 1995).

Although the preparation of fluorescent DNA and RNA can be achieved with a relatively standard procedure, conjugation of fluorescent probes to proteins remains an art due to the large variability in reactive properties among proteins and reagents. Therefore, an optimal condition has to be determined for each protein through trial and error. If the protein shows a very low reactivity toward a number of fluorescent reagents, it is often possible to create a reactive site in a nonessential domain by point mutagenesis (Tarachandani and Wang, 1996).

V. PITFALLS

The measured labeling ratio is limited by the accuracy of the extinction coefficient of the fluorophore. The value often changes upon reaction and could even vary among different lots of the reagent. Thus the measured labeling ratio should be treated only as an estimate.

Incorporation of too many fluorophores per RNA or protein can cause deleterious effects on the biological activity or cellular structures. The reaction condition should be adjusted to yield a 1:1 ratio for most proteins, and the biochemical activities of the conjugates should be checked carefully. The extent of RNA labeling can be controlled by using a ratio of 1:10 for modified rNTP:rNTP in the *in vitro* transcription reaction. This yields functional fluorescent RNA assayed to date. However, the limit in the extent of conjugation results in limited signals, particularly for small RNA molecules, and requires the use of very sensitive detectors (Jacobson *et al.*, 1995).

The spectrum and intensity of fluorescence can vary dramatically with the conformation and ligand interaction of both proteins and nucleic acids (Vamosi *et al.*, 1996). Spectroscopic characterization of the conjugate *in vitro* is critical for interpreting any experimental results involving quantitative measurements of fluorescence, such as ratio imaging and resonance energy transfer.

References

Adams, S. R., Harootunian, A. T., Buechler, Y. J., Taylor, S. S., and Tsien, R. Y. (1991). Fluorescent ratio imaging of cyclic AMP in single cells. *Nature (London)* 349, 694–697.

Jacobson, M. R., Cao, L. G., Wang, Y.-L., and Pederson, T. (1995). Dynamic localization of RNase MRP RNA in the nucleolus observed by fluorescent RNA cytochemistry in living cells. *J. Cell Biol.* 131, 1649–1658.

Langer, P. R., Waldrop, A. A., and Ward, D. C. (1981). Enzymatic synthesis of biotin-labeled polynucleotides: Novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. USA* 78, 6633–6637.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (eds.) (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Press, Cold Spring Harbor, NY.

Taneja, K. L., McCurrach, M., Schalling, M., Housman, D., and Singer, R. H. (1995). Foci of trinucleotide repeat transcripts in nuclei of myotonic dystrophy cells and tissues. *J. Cell Biol.* 128, 995–1002.

Tarachandani, A., and Wang, Y.-L. (1996). Site-directed mutagenesis enabled preparation of a functional fluorescent analog of profilin: Biochemical characterization and localization in living cells. *Cell Motil. Cytoskel.* 34, 313–323.

Theriot, J. A., and Mitchison, T. J. (1991). Actin microfilament dynamics in locomoting cells. *Nature (London)* 352, 126–131.

Varmosi, G., Gohlke, C., and Clegg, R. M. (1996). Fluorescence characteristics of 5-carboxytetramethylrhodamine linked covalently to the 5′ end of oligonucleotides: Multiple conformers of single-stranded and double-stranded dye-DNA complexes. *Biophys. J.* 71, 972–994.

Wang, Y.-L. (1985). Exchange of actin subunits at the leading edge of living fibroblasts: Possible role of treadmilling. *J. Cell Biol.* 101, 597–602.

Wang, Y.-L. (1989). Fluorescent analog cytochemistry. *Meth. Cell Biol.* 29, 1–12.

Wang, Y.-L. (1992). Fluorescence microscopic analysis of cytoskeletal organization and dynamics. *In* "The Cytoskeleton: A Practical Approach" (K. L. Carraway and C. A. C. Carraway, eds.), pp. 1–22. IRL Press, Oxford.