Expression of Green or Red Fluorescent Protein (GFP or DsRed) Linked Proteins in Nonmuscle and Muscle Cells

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Abstract

The introduction of the green fluorescent protein (GFP) plasmids that allow proteins and peptides to be expressed with a fluorescent tag has had a major impact on the field of cell biology. It has enabled the dynamics of a wide variety of proteins to be analyzed that could not otherwise be detected in live cells. Transient transfections of muscle and nonmuscle cells with plasmids encoding various cytoskeletal proteins ligated to green fluorescent protein or Ds red protein allow changes in the cytoskeletal network to be studied in the same cell for time periods up to several days. With this approach, proteins that could not be purified and directly labeled with fluorescent dyes and microinjected into cells can now be expressed and visualized in a wide variety of cells. Procedures are presented for transfection of the nonmuscle cell, PtK2, and primary cultures of embryonic chick myocytes, and for studying the live transfected cells.

Index Entries: Green fluorescent protein; Ds red protein; cardiac muscle; PtK2 cells, nonmuscle cells; actin; alpha-actinin; stress fibers; focal adhesions; lamellipodia; myofibrils; dense bodies; Z-bodies; Z-bands.

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1. Introduction

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One of the most useful tools to become available for biologists in the 1990s is the family of expression plasmids designed to ligate proteins and peptides to the green fluorescent protein (GFP) of the luminescent jellyfish, *Aequorea victoria* ***(1,2)***. Modifications made to the original expression plasmid for this jellyfish protein have enhanced the fluorescence to about 35 times brighter than the wild-type protein ***(3,4)***. The size of GFP, about 28 kD ***(5,6)***, creates a large fluorescent tag in comparison to the 500 Dalton fluorescent dyes (e.g., fluorescein or rhodamine) that previously have been coupled to purified proteins ***(7)***; nevertheless, GFP does not seem to interfere with most interactions of its linked protein. There are cases, however, where the placement of the large GFP probe has interfered with the nearby domains of proteins. For example, coupling GFP to the N-terminal region of alpha-actinin, near the actin-binding domain, resulted in a GFP probe that could not bind actin, whereas GFP linked to the C-terminus of alpha-actinin resulted in a probe that readily bound actin filaments ***(8)***. An alternate approach to eliminating interference by GFP is to introduce a spacer of 10 amino acids ***(9)*** or even a protein domain ***(10)*** between the GFP probe and the protein. The expression of GFP-linked proteins has particularly been advantageous for following proteins whose low abundance or solubility properties make them unsuitable for microinjection into living cells. The cDNAs for a number of abundant cytoskeletal proteins have also been coupled to GFPs because transfection

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can be easier than microinjection and cell lines can be created expressing these GFP-cytoskeletal proteins. The expression of GFP plasmids encoding different proteins has permitted the dynamic changes of the proteins to be studied in living cells during events such as cell division ***(11)***, cell locomotion ***(12),*** and myofibrillogenesis ***(8)***.

In addition to GFP, there are now plasmids encoding blue, cyan, and yellow variations of the basic product isolated from *A. victoria* ***(13)***. A new plasmid encoding a red fluorescent protein, called DsRed, from a nonbioluminescent coral (Discosoma species "Red"; DsRed) native to the Indo-Pacific area ***(14)***, has recently been released (Clontech, Palo Alto, CA). Although, from an unrelated organism, the molecule has the same beta-can shape and size as the jellyfish GFP. Double transfections of cells with different combinations of these plasmids allows two proteins to be followed at separate wavelengths in living cells.

This article outlines the steps involved in the transfection of cultured nonmuscle and muscle cells with expression plasmids coding for GFP, and DsRed. Descriptions are based on (a) the transfection of PtK2 cells, an epithelial renal cell line, with a plasmid encoding actin or alpha-actinin ligated to GFP and (b) the transfection of myocytes in primary cultures of quail skeletal muscle cells with GFP or DsRed ligated to alpha-actinin. Alpha-actinin is an actin-binding protein that crosslinks actin filaments, and is concentrated in the Z-bands of muscle cells and in focal adhesions, dense bodies of stress fibers, and lamellipodia of nonmuscle cells ***(8,15)***. The same procedures would be followed for other plasmids encoding GFPs or DsRed coupled to other host proteins.

2. Materials

2.1. Nonmuscle Cells

1. Cells: *Potorous tridactylis* (rat kangaroo) kidney cell line (PtK2, ATCC, Rockville, MD).
2. PtK2 medium: 10% fetal bovine serum (FBS, Life Technologies Inc., Gaithersburg, MD), 1% L-glutamine (200 m*M*, Life Technologies Inc.), in minimum essential medium with Earle's salts and without L-Glutamine (MEM, Life Technologies Inc.). Filter medium using a sterile filtration system (0.22 µm membrane, e.g., Stericup™, Millipore, Bedford, MA). Store in the refrigerator.
3. Serum-free medium: Opti-MEM® (Life Technologies Inc.).
4. Trypsin-EDTA (0.25%, Life Technologies Inc.).
5. Culture dishes:
   a. 35 mm glass bottom microwell dishes, No. 1.5, uncoated (MatTek, Ashland, MA).
   b. $22 \times 22 \times 1.5$ mm coverslip (Fisher Scientific, Pittsburgh, PA) placed in a 35 mm Petri dish (Fisher).
6. Liposomal transfection reagent: Lipofectin™ (Life Technologies Inc.).
7. Plasmids. Expression plasmids encoding GFP are available from a number of companies. We have used the plasmid encoding an enhanced GFP (Clontech). Standard molecular biology techniques are used to place the cDNA for GFP on either the N- or C-terminus of the selected protein.
8. Low-salt solution: 0.1 *M* KCl, 0.001 *M* $MgCl_2$, 0.006 *M* K_2HPO_4, 0.004 M KH_2PO_4, pH 7.0. Add the following stock solutions to 3 L of distilled water: 190 mL 2*M* KCl, 3.8 mL 1*M* $MgCl_2$, 23 mL 1 *M* K_2HPO_4, 15 mL 1 *M* KH_2PO_4. Adjust the pH to 7.0 and add additional distilled water to a total volume of 3.8 L.
9. Paraformaldehyde fixative: 3% paraformaldehyde in the low-salt solution**.** Dissolve 1.8 g NaCl in 150 mL of distilled water. Add 6 g of paraformaldehyde (e.g., "Baker" grade, J. T. Baker, Phillipsburg, NJ) to salt solution. Add three drops of 1 *M* NaOH from a Pasteur pipete. Heat to 60°C while stirring. After solution cools, add 1.6 mL of 1 *M* K_2HPO_4 and 0.4 mL of 1 *M* KH_2PO_4. Adjust pH to 7.4. Slowly and with stirring add 0.2 mL of 1 *M* $MgCl_2$ and 0.2 mL of 100 m*M* $CaCl_2$. Bring volume to 200 mL with distilled water. Aliquot and store at –20°C for 1 mo.
10. Permeabilization solution: 0.1% IGEPAL (Sigma, St. Louis, MO) in low-salt solution.
11. Blocking solution: 50 m*M* NH_4Cl in low-salt solution.

2.2. Muscle Cells

1. Cells: Primary culture of chick cardiomyocytes (from 8-d-old fertilzed chick eggs) and quail skeletal muscle cells (from 10-d-old fertilized quail eggs (Truslow Farms, Chestertown, MD).
2. Chick muscle medium: 10% FBS (Life Technologies Inc.) MEM (Life Technologies Inc.). Filter medium using a sterile filtration system (0.22 μm membrane, e.g., Stericup™, Millipore). Store in the refrigerator.
3. Quail muscle medium: 10% horse serum (HS, Life Technologies Inc.) 10% chick embryo extract (CE) ***(17)***, 1% L-glutamine (200 m*M*, Life Technologies Inc.), 1% antibiotic–antimycotic (Life Technologies Inc.), in MEM (Life Technologies Inc.). Filter medium using a sterile filtration system (0.22 mm membrane, e.g., Stericup', Millipore). Store in the refrigerator.
4. QM transfection medium: 5% HS, 3.3% CE, 1% L-Glutamine, in MEM without added L-Glutamine. Sterile filter. Store in the refrigerator.
5. Serum-free medium: Opti-MEM® (Life Technologies Inc.).
6. Culture dishes: Embryonic heart cells are grown directly on 35 mm microwell dishes with 1.5 mm glass bottom (MatTek, Ashland, MA). The quail skeletal myoblasts are grown on these dishes after they have been coated with rat tail collagen (Collaborative Biomedical Products, Bedford, MA), and left to dry and sterilize under UV light in the tissue culture hood overnight.
7. Liposomal transfection reagent: Lipofectin (Life Technologies Inc.).
8. Plasmids: Plasmid encoding DsRed (Clontech). Standard molecular biology techniques are used to place the cDNA for GFP or DsRed on either the N- or C-terminus of the selected protein.

3. Methods

3.1. Nonmuscle Cells

Cell culture and preparation for transfection: The day before transfection, subculture a confluent population of PtK2 cells onto either glass bottom dishes or coverslips (*see* **Notes 1** and **2**). To subculture, add 2 mL of trypsin to a 35 mm dish of confluent cells and incubate in a 37°C CO_2 incubator for 15 min. After 15 min, transfer the medium and detached cells to a sterile 14 mL snap-cap tube. Rinse the dish with an additional 2 mL of PtK2 medium to remove any loosely adherent cells and add this to the tube. Spin 5 min at setting 5 in a table top centrifuge. Decant the supernatant and resuspend the pellet in 6–8 mL of PtK2 medium. Distribute 1 mL of the suspension of cells to each of 6–8 culture dishes, and bring the total volume in each dish to 2 mL and incubate overnight This will yield 6–8 glass bottom dishes of approx 50% confluency the next day. Cells cultured on coverslips can be split in a similar ratio.

3.2. Muscle Cells

Cell culture of embryonic chick cardiomyocytes or quail myotubes and preparation for transfection:

1. Cardiac muscle cells are isolated from the hearts of 8- d-old chick embryos ***(17)***. The cells are plated at concentrations of 100,000 to 200,000 cells/mL. Transfect the cells 2 d after culturing.
2. Skeletal myoblasts are isolated from the breast muscles of quail embryos isolated from 10-d-old eggs *(17)*. The cells are plated at concentrations of about 100,000 cells/mL. The concentration can be varied depending on how dense a concentration of myotubes is desired. The number listed here is ideal for visualization of the ends and midsections of the growing myotubes. Transfect the skeletal muscle cells 1–2 d after culturing.

3.3. Transfection

1. Reagents**:** To each of two sterile tubes, add 200 μL of Opti-MEM for each dish of cells to be transfected. To one of the tubes, add 10 μL Lipofectin/dish to be transfected and to the other tube add 1–2 mg DNA for each dish (*see* **Notes 3, 4** and **5**). Incubate the two tubes at room temperature for 45 min. Mix the contents of the two tubes, vortex at a medium setting for 5 s, and incubate the mixture at room temperature for 15 min.
2. PtK2 or cardiac muscle cells: Add 600 μL of Opti-MEM/dish to be transfected to the tube of DNA–Lipofectin mixture and mix well. Remove the control medium (i.e., PtK2 medium or chick heart medium) from cells: swirl, and remove.

Repeat (*see* **Note 6**). Add 1 mL DNA–Lipofectin mixture to each dish of cells (*see* **Note 7**)**.** Incubate 3 h in 37ºC, 5% CO_2 incubator. Remove DNA-Lipofectin mixture from the cells. Add 2 ml control medium, swirl, and remove. Repeat. Add 2 mL control medium and return the cells to the incubator. Cells will reach optimal level of GFP expression in 24–48 h (**Figs. 1–3**).

3. Quail myotube primary culture: Add 600 µL QM transfection medium to the DNA–Lipofectin mixture and mix well. Remove QM medium from cells. Add 2 mL QM transfection medium, swirl, and remove. Repeat (*see* **Note 6**). Add 1 mL DNA-Lipofectin mixture to each dish of cells. Incubate 16–24 h in 37ºC, 5% CO_2 incubator. After incubation, remove the mixture from the dish. Add 2 mL QM transfection medium, swirl, and remove. Repeat. Add 2 mL QM medium (with antibiotics) and return to the 37ºC CO_2 incubator. Cells take at least 48 h to express DsRed fluorescence **(Fig. 4).** GFP-alpha-actinin can be detected in the Z-bodies of premyofibrils and Z-bands of mature myofibrils ***(8)*** over the next 2 d. This first generation DsRed plasmid, however, gives variable results. In some cells the DsRed-alpha-actinin, is bright, but not incorporated into any sarcomeric structure. With improved , DsRed will be very useful in combination with GFP probes.

3.4. Microscopy of Live Cells

1. Incubation of cells: The GFP fusion protein can be followed over the course of several days in cells grown on glass bottom dishes (**Figs. 1** and **2**). A heat curtain (ASI 400 Air Stream Incubator; NevTek, Burnsville, VA) is used to maintain a temperature of 37ºC on the stage of the microscope. To provide the cells with a 5% CO_2 environment, first make a small hole in the side of the bottom half of a 100 mm Petri dish by inserting a heated 22 gauge needle through the plastic. Fit a CO_2 regulator with an adapter that can be connected to rubber tubing which will end in a fitting for a 22-gauge needle that will deliver the gas into the inside the Petri dish (*see* **Note 8**). Invert the Petri dish over the dish of transfected cells and perfuse through CO_2 from a tank of 5% CO_2/balance air. A weight should be placed on the dish to hold it in place. An annular microscopic insert works well to hold down the dish and does not interfere with imaging. If the glass coverslips have been marked with a diamond-tipped scribe in a series of loops or a grid, the transfected cells can be returned to the incubator after an image is recorded and the cells then relocated for imaging at subsequent time points (see **Note 9**).
2. Imaging of transfected cells: GFP expression can be seen in less than 24 h after the start of transfection. In some instances expression can be seen as early as 5 h The transfected cells can be identified with epifluorescence optics and filters optimized for the species of GFP used (Chroma, Technology Corp., Brattleboro, VT; Omega Optical, Brattleboro, VT). The glass bottom dishes allow high N.A. oil immersion lenses to be used. It is best to use the minimum level of exciting light to avoid bleaching and damaging transfected cells. Images can be acquired with intensified video cameras or cooled CCD cameras keeping the exposures as brief as possible. Image processing software allows time-lapse recording to be automated and images saved to disk. Changes in the intensity of the GFP signal can be measured over time and correlated with changes in structure.
3. Fixing cells and counterstaining: The transfected cells can be fixed and permeabilized so that other cellular proteins can be identified with immunofluorescence. F-actin can also be detected by the use of various fluorescently labeled phalloidins ***(16)***. The GFP-alpha-actinin (or other localized GFP-labeled proteins) in the stress fibers, lamellipodia, or dense bodies are not lost during these processes.

   Fix the cells by incubating them in 2 mL of the paraformaldehyde solution for 15 min at room temperature. Rinse several times with the low salt solution. Permeabilize the cells with 2 mL of the permeabilization solution for 5 minutes at room temperature. Rinse again with the low-salt solution. Add 2 mL of the 50 m*M* NH_4Cl solution for 5 min at room temperature to block any unreacted aldehyde groups of the fixative. Rinse cells again with the low-salt solution. The cells are now ready to be stained with antibodies or phalloidin using standard techniques.

4. Notes

1. Do all cell culture work in a sterile environment, i.e., a tissue culture hood. Rinse hands and swab medium containers and tubes with 70% ethanol to reduce the chance of contaminating the culture. Care should be taken not only in the feeding and subculturing processes but also in the preparation of the medium and other solutions that are to be used with live cells. Coverslips can be sterilized by exposure to UV light overnight in the hood.
2. The glass bottom dishes are useful for high magnification viewing of the GFP signal in live cells over several hours or days. Coverslips are more convenient if cells are to be fixed and stained with an antibody. They can be mounted on a slide with a mounting agent (e.g., Mowiol, Boehringer Mannheim, Indianapolis IN).
3. A UV spectrophotometer can be used to quantify the amount of DNA in a solution. An optical density (OD) of 1 at a wavelength of 260 nm equals 0.05 μg/μL.
4. The amounts of Lipofectin and DNA added as well as the time of incubation of the mixture with the cells can be changed to try to optimize efficiency. The above amounts and times work very well and typically yield transfection rates with GFP-alpha-actinin of 40–60% for PtK2 cells and 100% for myotubes.
5. The purity of the DNA is also important for good transfection efficiency. The 260/280 nm absorbance ratio of DNA to protein should be 1.7 or above. Concert plasmid Maxi kits (Life Technologies Inc.) yield good quality DNA for use in transfections. An endotoxin-free plasmid maxi kit can be used to prepare DNA virtually free of lipopolysaccarides that have been shown to reduce transfection efficiency ***(18)***.
6. Lipofectin increases sensitivity of cells to antibiotics, so cells must be rinsed thoroughly to remove antibiotics before the DNA–Lipofectin mixture is added. The QM transfection medium used to prepare the DNA–Lipofectin mixture must not contain antibiotics.
7. The presence of serum reduces the efficiency of transfection. However, we have found that when transfecting PtK2 cells with Lipofectin, we achieve satisfactory transfection efficiencies without first rinsing the cells with Opti-MEM. Omitting the rinsing step significantly decreases cell death during and after transfection.
8. Plastic wrap can be placed over the stage with a hole for the objective lens to make a tighter seal to keep the CO_2 under the Petri dish. Humidity can be provided by two methods. Small containers (e.g. a cap of a tube) filled with water can be place under the dish to provide humidity for the cells. In addition, the CO_2 can be bubbled through distilled water before it reaches the needle. A tank of 5% CO_2/air accepts a regulator with a CGA-590 nozzle.
9. Sterilize the diamond-tipped pen before scoring the dishes. Expose the open dish to UV light for at least 1 h after making the marks to sterilize the dish. A suggested pattern of marks is three rows of loops each with a different number of loops.

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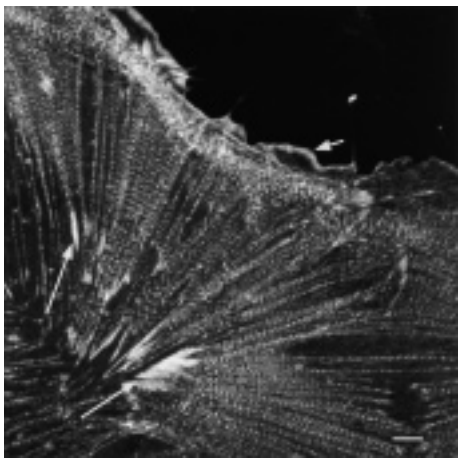

Fig. 1. PtK2 cell transfected with an enhanced GFP plasmid containing the cDNA encoding alpha-actinin. Note the localization of alpha-actinin-GFP in the lamellipodium on the left of the cell (short arrow), the focal adhesions (long arrows) and the linear banded dense bodies of the stress fibers. The spacings between these dense bodies are about 0.9 mm. Two adjacent fluorescent dense bodies along the stress fiber mark the boundaries of a single minisarcomere of the stress fiber (**19**). Similar banded arrays of fibers are also detected in the cleavage furrows of dividing cells (**20,21**). Bar = 5 mm.

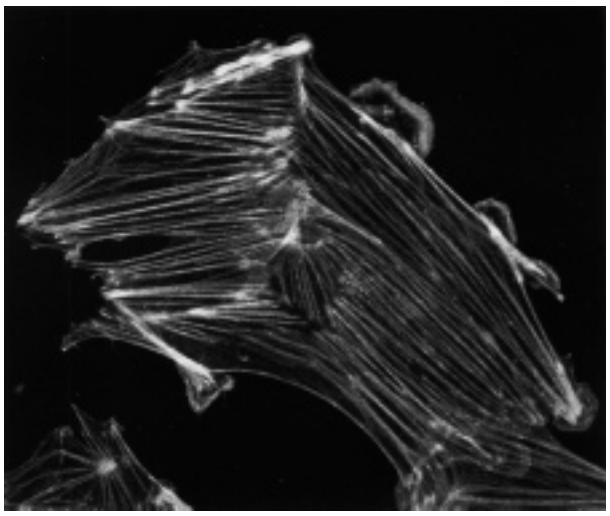

Fig. 2. PtK2 cell transfected with an enhanced GFP plasmid containing cDNA encoding actin. The actin localizes to the focal adhesions, lamellipodia, and the stress fibers.

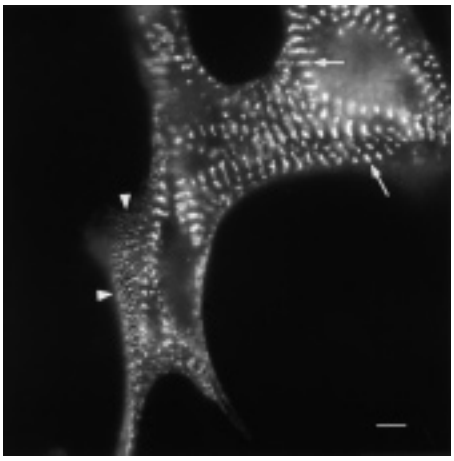

Fig. 3. Embryonic chick cardiomyocyte with a GFP plasmid containing the cDNA encoding alpha-actinin. Note the concentration of alpha-actinin-GFP in the Z-bodies of the premyofibrils at the edge of the spreading cardiomyocyte (arrowheads) and in the Z-bands of the mature myofibrils (arrows). The premyofibrils are the precursors to the mature myofibrils (**8**). The spacings between the Z-bands of the mature myofibrils are about 2 mm. Bar = 5 mm.

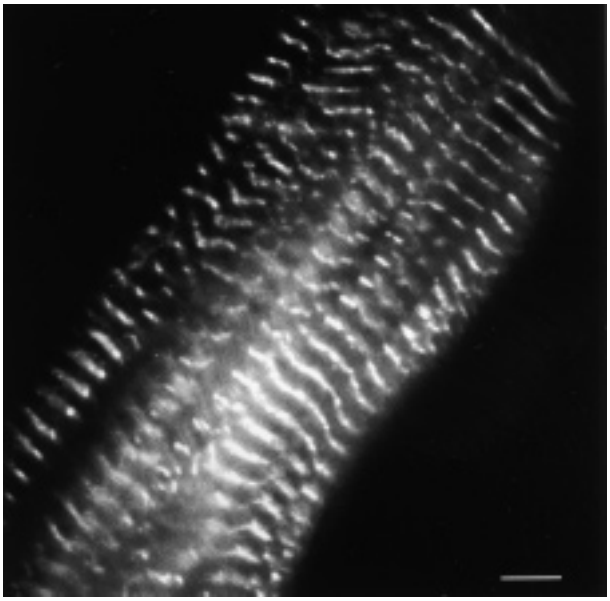

Fig. 4. Quail myotube transfected with a DsRed plasmid containing the cDNA encoding alpha-actinin. Note the concentration of alpha-actinin-DsRed in the Z-bands of the mature myofibrils. The spacings between these Z-bands are about 2 microns. Bar = 5 mm.